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The secretory IgA response to shigella antigens has been investigated in rabbits and mice. These studies established a major route by which shigella are taken up for antigen processing by the gut-associated lymphoid tissues. The uptake by M cells overlying lymphoid follicles in the gut may serve as a double-edged sword. Whereas this is the initial step in processing of the shigella antigens to develop a mucosal immune response, it is also a portal of entry through which pathogenic shigella prefer to pass and where they can replicate creating the ulcerations characteristic of human dysentery. The cellular basis for the secretory IgA response is being investigated. Present studies have shown how lymphocytes from several sources of gut-associated lymphoid tissue can be used to determine when an animal has been effectively primed for a secretory IgA memory response against *S. flexneri* lipopolysaccharide. Future studies will dissect the role of T-cells in the development of this response. We have established that a secretory IgA response against Shiga toxin can be elicited in our Thiry-Vella loop model in rabbits. Further, by using the antibodies from these studies in both *in vivo* and *in vitro* evaluations, we found that the IgA anti-Shiga toxin can protect against the toxic effects of that molecule. Much of the secretory IgA immune response is directed against the A and B subunits of Shiga toxin when a crude preparation containing many other antigens is used to immunize rabbits intraloop. A mouse model for following the secretory IgA memory

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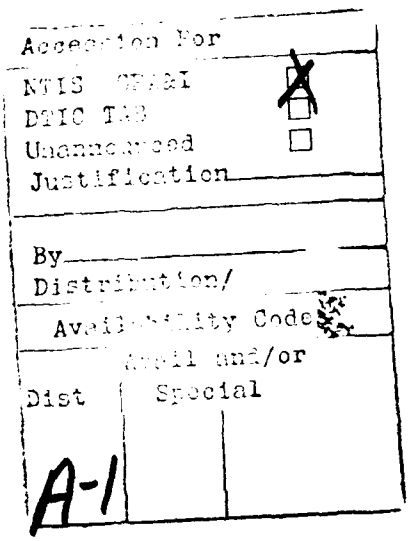
response to shigella antigens has been developed. Using Shiga toxin in this model, we have discovered it to be a particularly strong mucosal antigen which may be able to serve as an adjuvant to enhance the secretory IgA response against other antigens. The mouse model system will allow us to take advantage of the large library of monoclonal reagents and inbred strains to further our understanding of the basis for the secretory IgA memory response. Such an understanding is key to efficient development of future vaccines against shigella and other enteropathogens.



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INTRODUCTION

In order to provide basic information about the protective mucosal immune responses to relevant human pathogens, our laboratory has sought to establish a strong secretory IgA memory response in intestinal secretions against antigens present on or secreted by Shigella. To accomplish this goal, we have used our chronically isolated ileal (Thiry-Vella) loop model in rabbits to follow the kinetics of the IgA response (1). In previous studies using this rabbit model, we demonstrated the existence of a secretory IgA memory response against Shigella lipopolysaccharide antigens in intestinal secretions following appropriate priming (2). By taking advantage of the variety of strains of *Shigella flexneri* produced in the laboratory of Dr. Samuel B. Formal at the Walter Reed Army Institute of Research, our studies established that secretory IgA memory responses could be elicited by many different types of Shigella (3,4).

Early studies with the Thiry-Vella loop model of mucosal immunity to Shigella antigens used the hybrid strain of *S. flexneri* and *Escherichia coli* (*Shigella X16*) to establish that an IgA response could be elicited to nonpathogenic antigens and to determine the different methods of immunization required to stimulate the mucosal versus the systemic immune response. The *Shigella X16* strain invades the surface epithelium but does not reproduce once it is within host tissues, therefore, no ulceration is produced. By giving this bacterium directly into isolated intestinal loops, a strong local IgA response was produced (5). Similar responses were found with the invasive strain M4243 (which does cause ulceration) and with a noninvasive strain 2457-0 when applied directly into the isolated ileal loops. The presence of a Peyer's patch locally within the isolated loop and the dosage schedule were other important factors influencing development of the mucosal immune response (5). These early studies proved that when Thiry-Vella loops were stimulated directly with various Shigella preparations, secretions collected from those loops would contain considerable antigen-specific secretory IgA but little or no IgG directed against Shigella. This was not due to rapid degradation of IgG (which is normally destroyed quickly in intact intestine) since the isolated ileal loops were separated from the proteolytic effects of gastric acid, bile and digestive enzymes including trypsin, pepsin and chymotrypsin (6). Direct stimulation of the isolated loops by Shigella antigens resulted in little or no systemic IgG against Shigella unless the systemic immune response had been previously primed by a parenteral dose of Shigella (7). To elicit IgG in the serum, it was necessary to give the bacteria parenterally; that route resulted in virtually no secretory IgA anti-Shigella LPS.

To evaluate the secretory IgA memory response, we immunized rabbits orally with three doses of live or killed *Shigella flexneri* before the Thiry-Vella loops were created.. This more natural route of immunization was used to approximate the situation in vaccinating humans. After the animals rested for two months, a chronically isolated ileal loop was created. An oral challenge dose of live Shigella was given and secretions from the chronically isolated ileal loops were assayed for the secretory IgA anti-Shigella LPS response using an enzyme-linked immunosorbent assay (ELISA). The use of the Thiry-Vella loops as a probe was based on background information about lymphocyte trafficking after intraluminal antigen stimulation for a secretory IgA response. The initial step in stimulation of the secretory IgA response involves phagocytosis of antigens by specialized surface epithelial cells, M cells which are present within the epithelium overlying lymphoid structures throughout the gastrointestinal tract (8-10). Through these M cells, antigenic material, microorganisms and soluble proteins are brought into the underlying gut-associated lymphoid tissues (GALT). Once within GALT, these antigens come into contact with precursor B-lymphoblasts which are genetically predisposed to develop secretory IgA response (11).

Also within GALT, the B-lymphocytes come under the influence of specific regulatory cells, originally described as "switch T-cells" by Kawanishi et al. (12). The latter cells encourage B-lymphocytes to alter the phenotype of their surface heavy chain from mu chain to alpha chain. In addition, there are other helper T cells in GALT which help B-lymphocytes mature to IgA secreting plasma cells (13-15). Although the precise mechanism of this switch is unclear, it is known that interleukins 4 and 5 play a role in the process (16,17). Following their stimulation in GALT, these antigen-specific B-lymphoblasts migrate in turn to the mesenteric lymph nodes, the thoracic duct, and eventually lodge in the spleen where they undergo some degree of maturation (18,19). The final site of lodging by these B-lymphocytes may be somewhat influenced by the location of initial antigen stimulation (20). That is, when antigens are applied directly to the respiratory tract, the antigen-specific B-lymphocytes are more likely to return to that site than to the gastrointestinal tract or to mammary secretions. Conversely, when antigen is applied to sites in the gastrointestinal tract antigen-specific B-lymphocytes are more likely to return to the gastrointestinal tract than to other mucosal surfaces. In our studies of the mucosal memory response, we took advantage of this lymphocyte trafficking to use the chronically isolated ileal loops as a probe for the mucosal memory response. We found that live noninvasive shigella were as effective as virulent invasive strains of Shigella at eliciting a secretory IgA memory response against Shigella LPS (2-4).

Having established the existence of a secretory IgA memory response to Shigella LPS and the fact that invasive bacteria need not be used to elicit this response, we set five major goals for the present studies. Our first goal was to examine the mechanism by which the Shigella antigens were initially taken up by the GALT. These ultrastructural studies indicated that avirulent strains of Shigella (even heat-killed) were taken up by the follicle-associated epithelial M cells (21). However, virulent strains of *Shigella flexneri* were able to replicate once within this GALT epithelium resulting in destruction of the dome areas and ulceration of the epithelium. These focal lesions may serve as the initial sites where Shigella prefer to enter host tissues and spread laterally. The many isolated follicles present in the colon and ileum could serve as preferential sites of entry by this microorganism.

The second goal during the past year and a half has been to examine the cellular basis of this secretory IgA memory response. We have found that by the fourth day following oral rechallenge, sufficient numbers of antigen-specific IgA B-lymphoblasts are present in Peyer's patches and mesenteric lymph nodes that their secreted product in culture can be easily detected by ELISA. These cells were so consistently found in our early studies that we may be able to use their presence to infer establishment of a mucosal memory response. This will allow a much more rapid evaluation of new vaccine preparations as we could immunize large numbers of animals with potential vaccine strains (formerly, only one or two animals could be immunized per day due to the need to surgically create the Thiry-vella loops). After two months, the animals will be rechallenged orally and on the fourth day following challenge lymphocytes from the mesenteric lymph nodes and Peyer's patches will be grown *in vitro* to determine the capacity of the B-lymphocytes to form an IgA memory response against the vaccine preparation.

The third goal of these studies was to examine the various specificities of the secretory IgA response to Shigella antigens. For this, we used our rabbit loop system to examine the secretory IgA response against Shiga toxin. Extremely strong secretory IgA responses in intestinal secretions were elicited consistently by direct intraloop immunization with Shiga toxin preparations provided by Dr. J. Edward Brown. At the same time, systemic IgG responses are also elicited by this mucosal immunization route. The

secretions from this work were used as part of the studies on the functional significance of secretory IgA against shigella antigens (see below).

By Western blotting technique, we examined the heterogeneity of the IgA responses against Shiga toxin. Overall, most antibodies were directed both against the A and B subunits with the A subunit antibody predominating. Relatively weak activity was found against other antigens. The titer of the secretory IgA response to Shiga toxin was higher than that seen against any antigen previously used in our loop model system except for cholera toxin (22). There are similarities between Shiga toxin and cholera toxin. Both are heterodimers composed of A and B subunits; in both the multiple B subunits bind to specific receptors on host epithelium, and in both the single A subunit mediates the toxic effects of the molecule. These observations encourage the hypothesis that both cholera toxin and Shiga toxin share a common mechanism for activating the secretory IgA response. Since cholera toxin has been shown to serve as an adjuvant to stimulate the mucosal immune response against other antigens co-administered or conjugated to it, we will examine whether secretory IgA against Shiga toxin will also show such an effect. Demonstrating that Shiga toxin is a mucosal adjuvant with a mechanism of action similar to that of cholera toxin, will pave the way to develop mucosal immunity against virtually any antigenic determinant by using one of these two adjuvants as carrier protein. To this end, we have begun establishing a mouse model (see above) which will allow us to examine both the humoral and cellular basis of the secretory IgA response against Shiga toxin.

Our fourth goal for this project was to establish a mouse model to study mucosal immunity to Shigella antigens. For the mouse model of the secretory IgA response to Shiga toxin, we have used the Elson technique, previously described with cholera toxin (23). Our preliminary studies indicate that a consistent secretory IgA response is elicited in the secretions with as little as 0.1 ml of crude Shiga toxin administered orally. The animals also demonstrate a strong IgG anti-Shiga toxin response in the serum. Relatively little IgM anti-Shiga toxin has been found. Future studies will concentrate on the IgA responses in intestinal secretions. The cellular basis of this response will be easier to study in the mice than in our rabbit model, as many monoclonal antibody preparations specific for mouse helper and suppressor T-lymphocyte populations are available. This will greatly facilitate future studies on the secretory IgA response against these Shigella antigens.

The fifth goal of the present work was to evaluate the biologic role of secretory IgA responses against shigella antigens. For these studies, we examined two possible modes of protection. The first involved using ligated loops of intestine to determine whether secretory IgA anti-Shigella LPS from the animals immunized orally with live Shigella would alter the uptake of the live bacteria by GALT. For the second, we examined both *in vitro* and *in vivo* model systems to determine whether secretory IgA against Shiga toxin is able to protect from the damaging effects of this molecule in a dose dependent fashion (24). The former studies have not been successful to date, whereas the latter have shown secretory IgA to be most effective in preventing the effects of Shiga toxin. While preliminary studies showed that animals receiving as many as five or six immunizations had a decrease in the number of Shigella taken up by the GALT, a large series of studies in which three oral immunizations were used, showed no significant difference in the uptake of Shigella by antigen-specific secretory IgA. The current protection models are inadequate to show uptake in a natural situation as they currently require us to use as many as 10^9 bacteria which likely overwhelm the immune response. In the natural infection, it is highly unlikely that numbers anywhere near 10^9 cause the disease. However, in our experimental models it has not been possible for us to have reproducible uptake of bacteria with fewer microorganisms than this. In the future, by using the RITARD model, we may be able to better approximate the natural infection requiring many fewer microorganisms to provide

reproducible bacterial uptake. The studies on the ability of secretory IgA against Shiga toxin to inhibit the toxicity of that molecule have been very successful. Both in vitro using the HeLa cell assay and *in vivo*, using acute, ligated loops of intestine in rabbits, IgA anti-shiga toxin has been able to inhibit the effects of Shiga toxin. Future studies will be directed to establishing an effective method to elicit a mucosal memory response to Shiga toxin to determine the duration of the protection achieved.

These studies continue to expand our understanding of the importance of the mucosal immune response in protection against enteropathogens and their toxic products. By exploring the precise modes of stimulation, the types of antigen and adjuvants which will consistently elicit mucosal memory responses and learning the cellular basis for these responses, we will be able to provide effective vaccines against many types of debilitating infectious diseases of mucosal surfaces.

BODY

I. METHODS

Preparation of Chronically Isolated Ileal Loops in Rabbits. The surgical creation of chronically isolated ileal loops in rabbits has been described in detail previously (1). Briefly, 3 kg New Zealand white rabbits (specific pathogen free) are anesthetized with xylazine and ketamine. A midline abdominal incision is made and the terminal ileum is identified. 20 cm of ileum containing a Peyer's patch is isolated with its vascular supply intact. Silastic tubing (Dow-Corning) is sewn into each end of the isolated segment. The free ends of the tubing are brought out through the midline incision and are tunnelled subcutaneously to the nape of the neck where they are exteriorized and secured. Intestinal continuity is restored by an end-to-end anastomosis. The midline incision is closed in two layers. Each day, about 2-4 ml of secretions and mucus that collect in the ileal loops are expelled by injecting 20 ml of air into one of the silastic tubes. Mucus is separated from the clear supernatant by centrifugation. The supernatant is stored at -20°C and assayed for specific immunoglobulin content. Care of the isolated loops requires a subsequent flush with 20 ml of sterile saline to remove adherent mucus. This saline is removed by repeated gentle flushes of air. With proper daily care, > 90% of our rabbits have completed experiments lasting for 2 months.

Mouse Lavage Model for Mucosal Immunity. The mouse model system is an adaption of one used by Elson for studies of the secretory IgA response to cholera toxin. For these studies, specific pathogen free mice were given 4 oral doses of lavage solution 15 minutes apart. Thirty minutes after the final lavage dose, a single intraperitoneal dose of 0.1mg pilocarpine was given. Pilocarpine encourages the mouse intestine to secrete large volumes of fluid. The mice are placed on wire mesh over beakers containing 3ml of protease inhibitor solution (soybean trypsin inhibitor in 50mM EDTA). Intestinal fluid and feces, and saliva, were collected over 30 minutes. The fluid collected is brought up to 5 ml volume with PBS and vortexed. After centrifuging the fluid at 650 X g for 10 minutes, 10µl of 100mM PMSF in 95% ethanol were added per ml of supernatant. The sample was then centrifuged 27,000 X g at 4°C for 20 minutes and the supernatant was saved. Again 10ul of PMSF in 95% ethanol and 10µl of 1% NaN_3 were added along with 50ul of fetal calf serum to each ml of the final supernatant. Samples were stored at -20°C until time of assay. Blood samples were taken from the animals by retroorbital bleeding.

Enzyme-linked Immunosorbent Assay (ELISA). Microtiter wells are coated with a solution containing the antigen of interest. For studies on Shiga toxin, we use the purified Shiga toxin preparation (see below) and for experiments on Shigella antigens, we have used a Westphal (hot phenol-water) extraction from *Shigella flexneri*. Concentration of the coating antigen is empirically determined with each new preparation of antigen. Standard IgG and IgA positive and negative samples are run with each new antigen preparation to ensure continuity from one lot to the next. Immediately prior to testing serum samples, loop secretions or mouse intestinal secretions, the antigen solution is removed and wells are washed with a phosphate-buffered saline solution (PBS) containing 0.05% Tween 20 (PT). The fluid to be assayed is diluted in the PT buffer and incubated in the coated and uncoated wells (the latter control for nonspecific adsorption) for 4 hours. The plates are washed with PT and incubated with either alkaline phosphatase-conjugated sheep anti-rabbit IgA, sheep anti-rabbit IgG (both isotype specific via affinity column purification in our laboratory as previously described (25)), or monoclonal anti-mouse IgG, IgA or IgM conjugated with alkaline phosphatase (Cappel). After a second incubation of 4 hours, the wells are again washed with PT and the substrate reaction is carried out with p-nitrophenyl phosphate in carbonate buffer pH 9.8. The kinetics of the enzyme-substrate reaction are extrapolated to 100 minutes. For the Shiga toxin assays, results are expressed as titer

using a standard as previously described (26). Specific IgG and IgA standards are processed on each plate with the unknown fluids as previously described (25,26). The data are analyzed using the RS1 integrated software system. Data are presented as titers or as geometric means. For each day's results, the variance is expressed together with the mean.

Antigen Preparations Used. Two major preparations of *Shigella flexneri* are used in the present studies: 1) *Shigella flexneri* M4243A$_1$. This strain lacks the 140 megadalton virulence plasmid and is not able to invade the surface epithelium. 2) *Shigella* X16. This is a hybrid of *S. flexneri* and *E. coli* which is able to invade the mucosa, does possess the 140 megadalton virulence plasmid, but does not persist within the epithelium following uptake *. Shiga toxin antigen preparations include a crude and purified preparation provided by Dr. J. Edward Brown. The crude preparation is a lysate of *Shigella dysenteriae* type 1 which has been passed over a diethylaminoethyl cellulose column. This preparation had 10^{4-5} CD_{50}/ml. The purified preparation, also provided by Dr. Brown, has been affinity- purified as described previously (27). Polyacrylamide gel electrophoresis and Western blotting of this preparation discloses two major bands consisting of the A and B subunits. A minor band, possibly a fragment of the A subunit, has been found on Western blotting of this preparation with specific antibodies made against the crude preparation.

In Vivo Assay for Uptake of Shigella by Follicle-Associated Epithelium and Villi. To determine the relationship between the virulence of the microorganism and the uptake of the bacteria by the follicle-associated epithelium, an *in vivo* assay procedure was employed. Isolated acute ileal loops 10 cm in length were created in specific pathogen free New Zealand bred white rabbits. A single dose containing 2×10^9 *Shigella flexneri* was injected into this acute loop. At 90 minutes and at 18 hours, these loops were removed and samples were fixed for histologic investigation by electron microscopy and light microscopy. For light microscopy, the sections were fixed in absolute ethanol and stained with Giemsa. For each time period, at least 10 sections of Peyer's patch and adjacent villi were examined for attachment and uptake of the Shigella. Histologically, these sections were divided into 2 areas: 1) the follicle-associated epithelium overlying the dome areas in Peyer's patches (known to be enriched in "M" cells); 2) villi which were outside of the Peyer's patch area. Evaluation was performed using oil immersion light microscopy. Since the normal flora of the rabbit ileum contains $< 10^4$ microorganisms, for statistical purposes, < 0.01% of the flora visualized were from other microorganisms. Further, the Shigella have a characteristic size and shape which under the circumstances of the this study were readily recognizable. The Bioquant Biometrics Image Analyzer (Nashville, Tennessee) with an IBM computer was used to measure the actual length in millimeters of the lining epithelium over the villi and over the dome regions of the Peyer's patches. The average of 100 fields for dome and villus areas from representative rabbits was calculated. This allowed us to express data as bacteria/mm^2 of surface epithelium. Therefore, a direct relationship of villus surface area to follicle-associated epithelium surface area was established, allowing for comparisons. Electron microscopy was performed on some sections demonstrating the characteristic rod-shaped structure and typical "M" cell location.

Mononuclear Cell Isolation. At time of sacrifice, rabbits from various immunization groups had peripheral blood, Peyer's patches, mesenteric lymph nodes, spleen and axillary lymph nodes removed under aseptic conditions. For the peripheral blood, the buffy coat was placed on lymphocyte separation medium and centrifuged at 400 x g at room temperature for 30 minutes. The cells at the interface were removed,

characterized and used as mononuclear cell preparations. Tissues were cut into 1 cm^3 fragments with a sterile blade and placed on sterile wire mesh. The cells were carefully teased apart and passed through steel mesh. This material was centrifuged at 400 x g at room temperature for 7 minutes. The pellet was gently resuspended and washed twice in RPMI 1640 medium. The total cells and viability were determined and a Wright stain preparation was examined to secure the differential count of the isolated cells.

In Vitro Mononuclear Cell Cultures. 10^5 mononuclear cells were added to each row of a 96 well, polystyrene microtest III tissue culture plate with flat bottom wells (Becton Dickenson). Cultures were placed in a humidified, 5% CO_2 37°C incubator. At the times indicated in the result section, the supernatant medium from 3 wells for each tissue was aspirated, and cell debris was removed by centrifugation at 400 x g for 15 minutes, and the supernatants were stored at -20°C. Assays were performed using the above described ELISA technique for Shigella LPS.

Electron Microscopy. Tissues for study were minced to approximately 1 mm^3 and fixed in 3% gluteraldahyde-formaldehyde in 0.1 M cacodylate buffer, pH 7.3. The samples were postfixed in 2% osmium tetroxide. After staining en block with 2% uranyl acetate, tissues were dehydrated in alcohol and embedded in epon. One micron thick sections were cut and stained with toluidine blue and examined for uptake of Shigella. Follicle-associated epithelial cells which contained Shigella were identified and thin sections (approximately 800 Angstrom's thick) were cut from these areas on a Porter-Blum MT-2 ultramicrotome. These thin sections were stained with lead citrate and examined with a Zeiss 109 transmission electron microscope. Photomicrographs were taken of the characteristic rod-shaped bacilli in the M cells.

Acute Loop Protection Studies. New Zealand white rabbits (specific pathogen free) are anesthetized with xylazine and ketamine. A midline abdominal incision is made and the terminal ileum is identified. A series of 5 cm segments from the mid-jejunum to the mid-ileum were created. Double 4.0 silk ligatures were placed between each segment to prevent leakage from one segment to another. Solutions to be tested for toxin or antitoxin activity were injected into the loops in the doses indicated. The midline incision was closed in two layers and the animals were allowed to rest for 18 hours. At time of sacrifice, the fluid contents of the loops were measured.

Cytotoxicity Assay. Shiga toxin activity was determined by examining the extent of HeLa cell damage by a previously described assay (28). Briefly, HeLa cell monolayers were grown in 96 well microtiter plates. For the assay, a standard crude toxin lysate of *S. dysenteriae* was incubated with serial dilutions of loop fluids for 30 minutes at room temperature. This mixture was placed onto the HeLa cell monolayer and allowed to incubate overnight at room temperature. The monolayers were then stained with crystal violet dissolved in 50% ethanol-1% sodium dodecyl sulfate, and the O.D. 620 nm was determined for each well. The dye remaining in each well correlates with the percentage of cell remaining adherent to the microtiter dishes. O.D. 620 nm of wells containing the standard toxin alone were averaged and that value plus two standard deviations was defined as the end point titer of loop fluids for neutralization of the cytotoxicity of the toxin preparation. All dilutions of loop fluid which gave an O.D. 620 in the assay greater than this value were scored as positive.

Immunodetection on Nitrocellulose Blots. Samples are subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis according to Laemmli (29). The initial

gel contains 12.6% acrylamide with a 3% acrylamide stacking gel. Molecular weight markers are applied to the gel as well as the sample of interest. Electrophoretic transfer of proteins is carried out as described by Towbin et al. (30) and Burnette (31). Proteins are electrophoretically transferred to nitrocellulose paper for 2.5 hours at constant voltage (60 V). After transfer, reactive sites remaining on the nitrocellulose are blocked by soaking the paper in tris-buffered saline containing .05% Tween 20 and 3% gelatin. This paper is reacted for 1 hour at 37°C with strips coated with either serum or loop fluid with reactivity to Shiga toxin. Following a gentle wash procedure, alkaline phosphatase-conjugated antibodies monospecific for rabbit IgG or IgA are added for 1 hour at 37°C. The substrate reaction allows visualization of the specific bands.

II. Results and Discussion

A. Studies on the Initial Antigen Processing of Different Shigella Preparations by the Intestine. The first goal of the present studies was to complete our examination of the initial uptake of different preparations of *Shigella flexneri* by the intestine. In the previous study period, we reported results of the uptake of live bacteria; now, the studies using heat-killed bacteria have been completed. With this work, we sought to determine how *S. flexneri* were taken up by the surface M cells which overlie lymphoid follicles in the gut. This initial uptake is important for development of the mucosal immune response to the Shigella antigens.

For these studies, segments of intestine were isolated as outlined in the methods section. Each group contained five rabbits (Table 1) to ensure reproducibility of the model. The bacteria listed in Table 1 were injected directly into each loop at time zero and at 30 minutes, 90 minutes and 18 hours; acute loops were removed and examined by both electron and light microscopy.

Table 1. Strains of Shigella flexneri Used for M Cell Uptake Studies in Rabbit Loops

Rabbit Group	S. flexneri strain	Plasmid[1]	Ulcer[2]	M-Cell[3]	Sereny[4]
1	M4243	+	+	+	+
2	X16	+	-	+	-
3	2457-O	+	-	+	-
4	M4243A1	-	-	+	-
5	Heat-Killed M4243	+	-	+	-

1. Presence of 140 megadalton virulence plasmid.
2. Mucosal ulceration produced in acute loops.
3. Uptake by M-cell demonstrated ultrastructurally.
4. Virulence demonstrated by Sereny test in guinea pigs.

For light microscopy, frozen sections were cut at 4 microns and stained with Giemsa. Ten sections were cut from each loop, for each time period. Therefore, 50 slides for each strain of bacteria, for each time period, were examined. All the slides were coded so that the observer would not know the time point or the particular animal or strain of shigella being examined. The number of shigella within both the follicle-associated epithelium overlying Peyer's patches and the villus epithelium were counted.

To determine the number of bacteria per unit area, the specimens were standardized using a Bioquant image analyzer. This allowed us to express our results as number of bacteria taken-up per micron of epithelium (figure 1). It also allowed us to correlate the uptake over isolated follicles with that over surface epithelium.

Figure 1. Photograph of the measurement of a dome region on the Bioquant image analyzer.

As shown in figure 2, this work has indicated that heat-killed Shigella were taken-up to a similar extent as that of nonpathogenic strains of shigella. On average,1.6 bacteria/mm^2 of surface epithelium were found in the follicle-associated epithelium, while ten-fold fewer bacteria were present in the adjacent villus epithelium. Therefore, the follicle-associated epithelium preferentially ingests both live and killed shigella. No evidence of ulceration was seen in any of the acute loops given the heat-killed shigella.

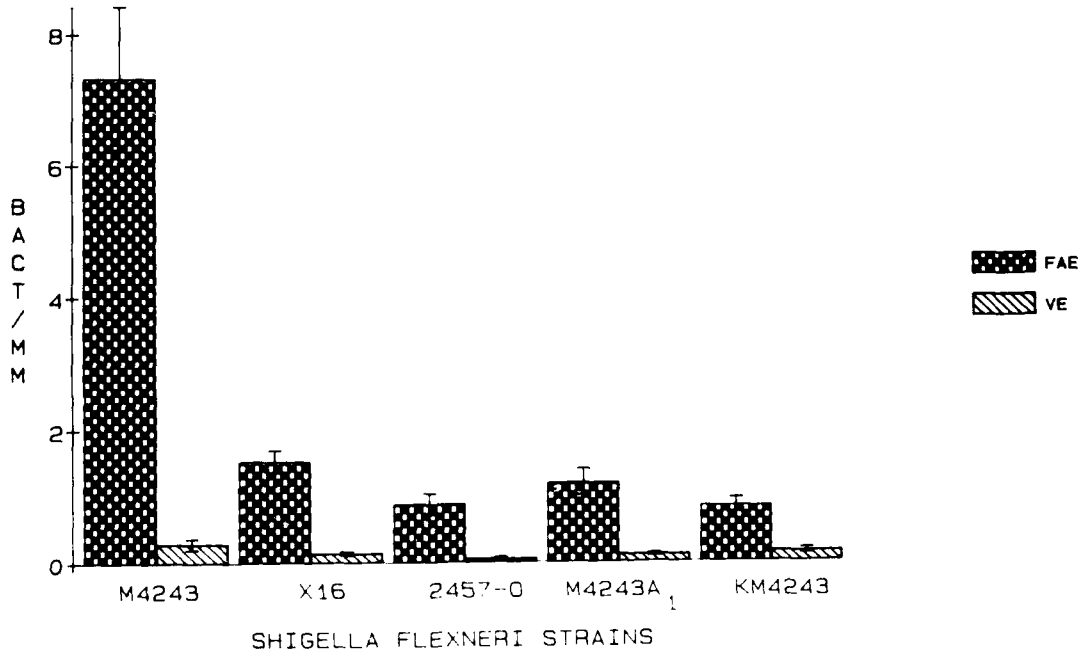

Figure 2. Uptake of S. flexneri measured on image analyzer. Follicle associated epithelium (FAE) over dome regions and villus epithelium (VE) were measured separately.

The presence of the bacteria in M cells was confirmed by examining electron microscopic fields of both villus and follicle-associated epithelium. Because light microscopy had shown that the major uptake of Shigella had occurred by 90 minutes, samples from this time period were examined. Denatured (heat-killed) Shigella were found within phagocytic vesicles of M cells in the follicle-associated epithelium.

The tissue damage at the 18 hour time period was correlated with the degree of uptake by the M cells in the follicle-associated epithelium (figure 3). All of the rabbits given pathogenic *S. flexneri* M4243 had ulcerations of isolated follicles and Peyer's patch follicles at 18 hours. In marked contrast, none of the animals given nonpathogenic or heat-killed Shigella had evidence of ulceration or even microulceration at this time.

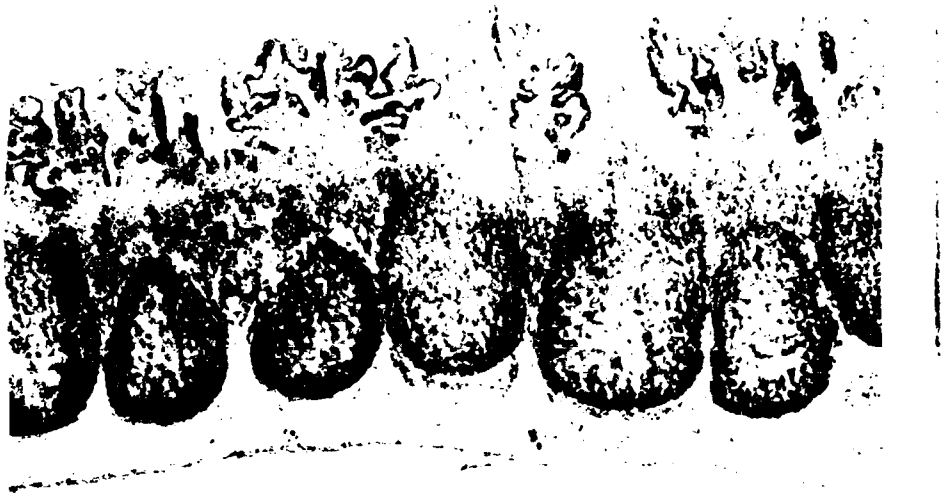

Figure 3. Follicle-associated epithelium with ulcerations over each dome area from the 18 hour study on a rabbit given *S. flexneri* M4243. Adjacent villi, though damaged are mainly intact.

In the acute loops given pathogenic *S. flexneri* M4243, evidence of damage to the villus epithelium was observed, but, no ulcerations were seen. There was mucus depletion and occasional focal acute inflammation. These findings indicated that *S. flexneri* is initially engulfed by the M cells wherein they multiply and eventually result in focal ulceration. Both nonpathogenic strains and heat-killed strains are processed by the M cells, which may explain the ability of these nonpathogenic strains to elicit a mucosal immune response against the *S. flexneri* LPS antigens. It is not clear why the heat-killed strain, which is taken-up in similar numbers as the live nonpathogenic strains, has proven to be incapable of priming animals for a mucosal memory response in our previous studies (2,3). It may be that the heat treatment damages antigens necessary for the appropriate stimulation of mucosal immunity.

B. Location of Shigella Antigen-specific Lymphocytes following Oral and Parenteral Priming. Our past studies have established that oral priming with either live pathogenic or nonpathogenic Shigella will consistently result in the stimulation of a secretory IgA memory response to subsequent oral challenge with live Shigella (2,3). Unfortunately, performance of these studies is a slow process requiring surgical preparation of chronically isolated ileal loops in rabbits and following the immune responses from these animals for several months. Only one or two rabbits can be prepared daily, and all the animals require daily care; this limits to 20-30 the number of animals which one can have in the colony. If we had a better understanding of the cellular basis of the mucosal memory response, it would be possible to streamline the testing of new potential vaccine strains by looking only at the specific cellular responses. To this end we have begun studies to determine the cellular basis of the secretory IgA memory response to *S. flexneri* antigens.

There is relatively little information available on the growth of rabbit gut-associated lymphoid cells *in vitro*, and the production of antigen-specific IgA by these cells. Therefore, we wished to explore both the native production of IgA anti-Shigella LPS by these cells and the effect of common mitogens such as PHA and PWM on this response. Our previous project had established the optimal concentrations of Con A and PWM to use in these studies.

Previous studies had shown that following three oral doses of the noninvasive *S. flexneri* M4243A1 (lacking the 140 megadalton virulence plasmid), animals were primed for a mucosal memory response (32) The rabbits were then allowed to rest for 60 days. A single oral challenge dose with the same bacteria was give, and on days 1, 3, 4, 5, 6, and 10 following this, the rabbits were sacrificed and the Peyer's patches, mesenteric lymph nodes, and spleen were separated into single cell suspensions. These suspensions were grown in tissue culture with ow without concanavalin A or pokeweed mitogen (using the previously determined optimal mitogenic doses). These cultures were maintained for as long as 3 weeks with samples of the supernatants assayed at various days to determine the specific immunoglobulin production. In addition, a procedure was set up on the RS1 software system for handling the large volume of data which was generated by these studies.

By day 3 following oral challenge with the live *S. flexneri* M4243A1, the lymphocytes from the Peyer's patches and the mesenteric lymph nodes contained considerable amounts of IgA anti-Shigella LPS (figures 4-7). Only lymphocytes from the spleen were found to produce significant amounts of IgG anti-Shigella LPS. Corresponding cells from control rabbits given only the single oral challenge prior to sacrifice produced virtually no IgA or IgG directed against Shigella LPS in cultures of the Peyer's patches, mesenteric lymph nodes, spleen or peripheral blood.

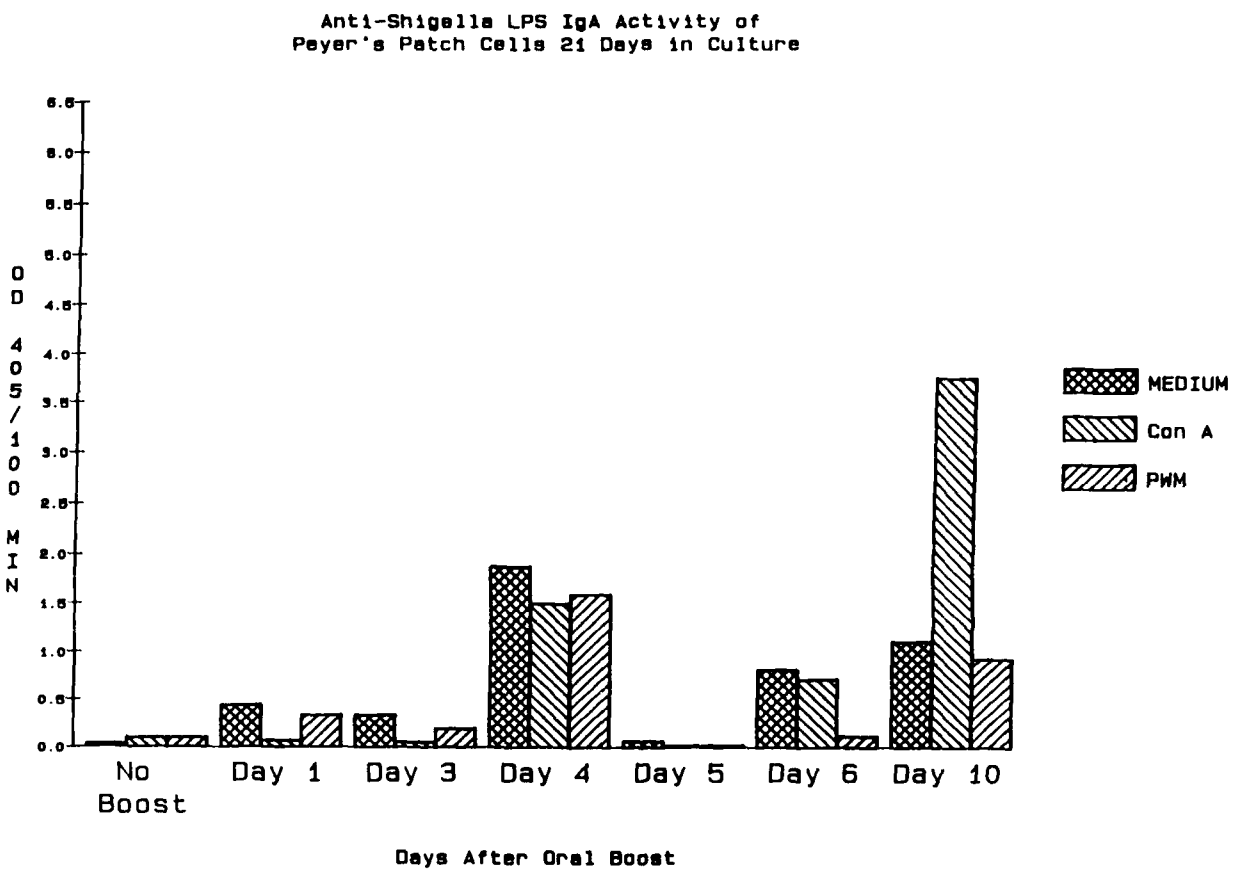

Figure 4. IgA anti-Shigella LPS response in culture supernatants from Peyer's Patch lymphocytes removed on the indicated day after oral challenge with live *S. flenxeri*. Responses in medium only, or with mitogens Con A or Pokeweed mitogen (PWM are noted).

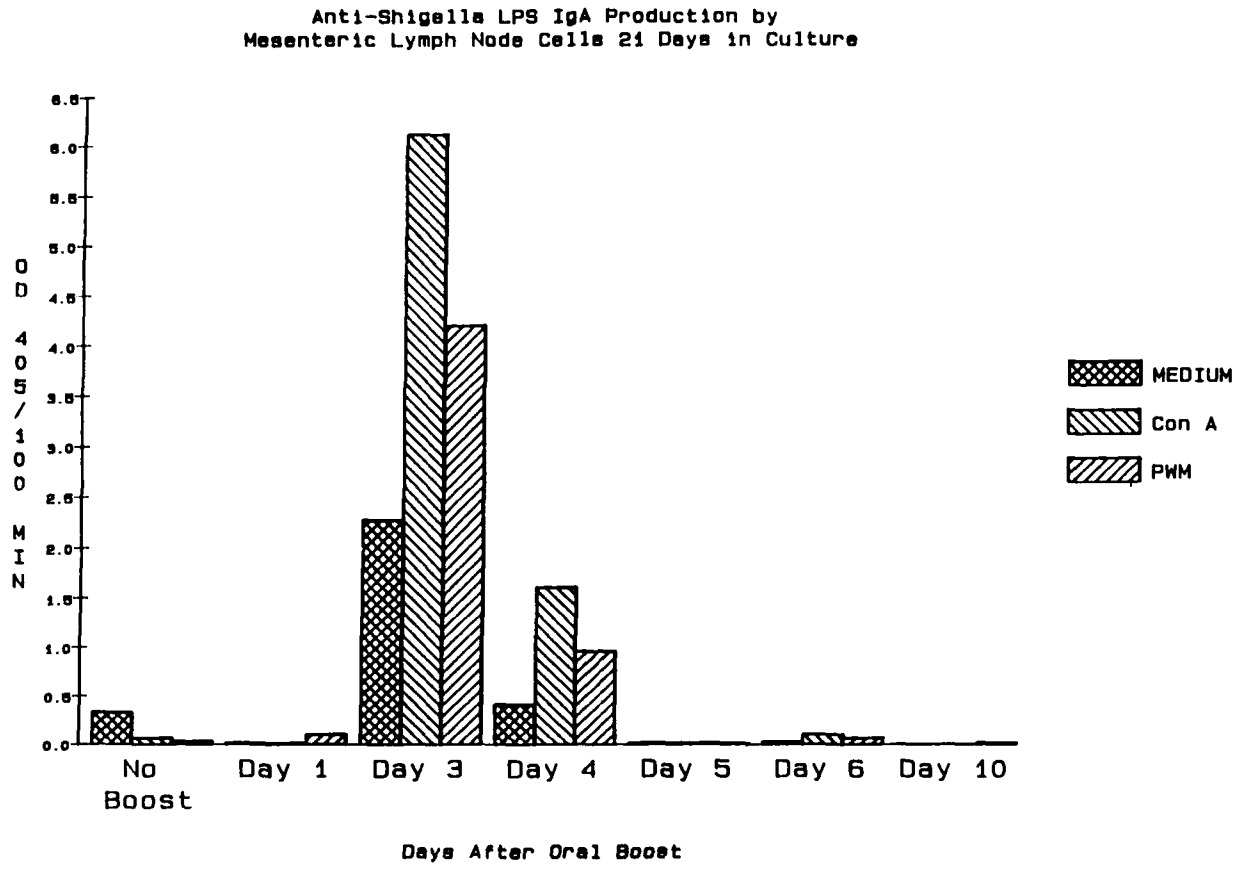

Figure 5. IgA anti-Shigella LPS response in culture supernatants from mesenteric lymph node lymphocytes removed on the indicated day after oral challenge with live *S. flexneri*. Responses in medium only, or with mitogens Con A or Pokeweed mitogen (PWM are noted).

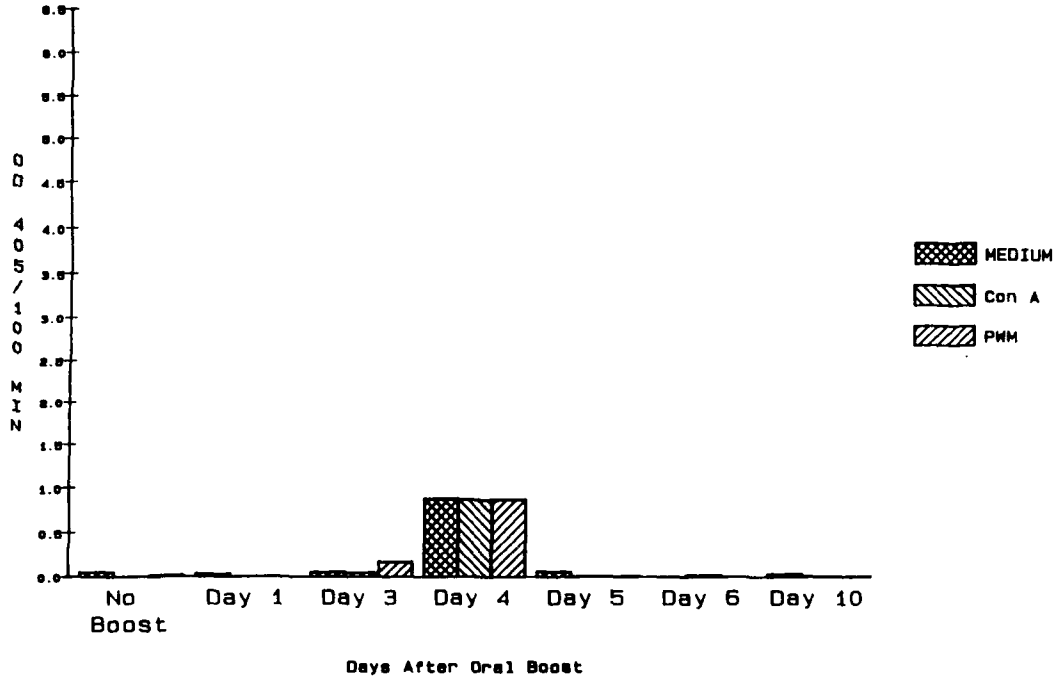

Figure 6. IgA anti-Shigella LPS response in culture supernatants from splenic lymphocytes removed on the indicated day after oral challenge with live *S. flenxeri*. Responses in medium only, or with mitogens Con A or Pokeweed mitogen (PWM are noted).

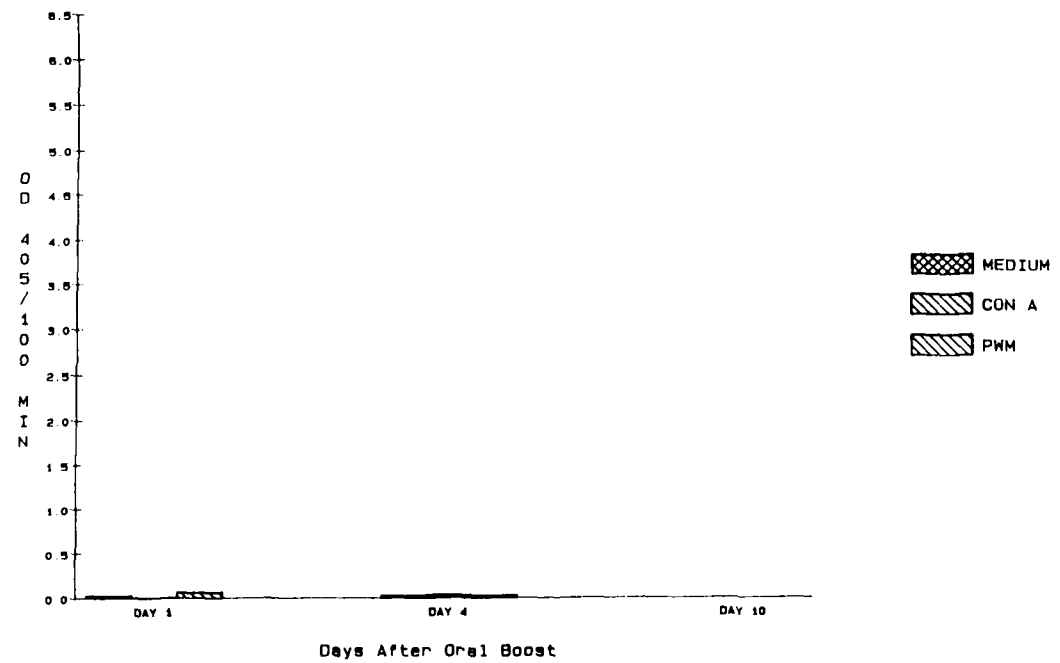

Figure 7. IgA anti-Shigella LPS response in culture supernatants from peripheral blood lymphocytes removed on the indicated day after oral challenge with live *S. flenxeri*. Responses in medium only, or with mitogens Con A or Pokeweed mitogen (PWM are noted).

The mitogens gave inconsistent results in our studies. In some rabbits, the mitogens gave an increase in the IgA response, such as the day 10 response from the Peyer's patches shown in figure 4. However, sometimes, the mitogens gave a weaker response such as the day 6 PWM response for the Peyer's patch cells. In one group of rabbits, we performed a mitogen dose response study for Con A and PWM on the specific IgA responses of their cell populations. Assays of cell supernatants from cultures receiving doses of Con A from 0.1 to 1.0 μg/ml, showed no specific pattern of reactivity. On day 14 in culture, the greatest anti-Shigella LPS response was seen with 5 μg/ml. We believe that the mitogens are probably not having a significant effect on the system and the variation we see is just reflecting the heterogeneity of the lymphoid population itself. Therefore, we have decided to eliminate mitogens from future cultures in favor of having more cells for replicate cultures. This will allow us to better estimate the variation between cultures in an individual rabbit.

A second series of rabbits was immunized with the same triple oral dose of live *S. flexneri* M4243A1 that the first group received. We wished to determine the degree of reproducibility of this *in vitro* assay system. This second group of rabbits showed the same overall reactivity of the Peyer's patch and mesenteric lymph node cells on day 4 following the subsequent oral rechallenge. This indicates that the in vitro model would be a reasonable method for use in quickly and efficiently evaluating the potential of vaccine strains of Shigella to develop a secretory IgA memory response.

C. Heterogeneity of Mucosal and Systemic anti-Shigella Responses. In the past, we have exclusively used ELISA on Shigella LPS (Westphal preparations) to study the secretory IgA responses. This technique limits our ability to have a detailed understanding of the key antigens involved in eliciting a strong secretory IgA response. Therefore, in the present studies on anti-Shigella LPS and anti-Shiga toxin responses, we will be using the Western blot assay to better determine key antigenic determinants which may be responsible for the secretory IgA responses seen.

During this period, we have developed the SDS-polyacrylamide gel electrophoresis and Western blot techniques for use with our model systems. We have developed our own mixture of molecular weight standards for the gels which include: lysozyme, beta-lactoglobulin, carbonic anhydrase, ovalbumin, bovine serum albumin and phosphorylase b. These cover the range of molecular weights from 14,000 to 96,000. The bacterial cell antigen preparations are made by boiling the cells for 5 minutes in phosphate buffer containing 1%d SDS, 10% glycerol and 5μM PMSF.

We have used this assay in recently completed studies on the IgA response to Shiga toxin. As shown in figure 8, this response is mainly against the A and B subunits, although other reactivities are seen. The prominence of the reaction against the A and B subunits suggests that Shiga toxin may be a particularly strong mucosal stimulant. As discussed in the next section, the strength of the mucosal immune response to Shiga toxin is matched only by previous studies with cholera toxin in our laboratory.

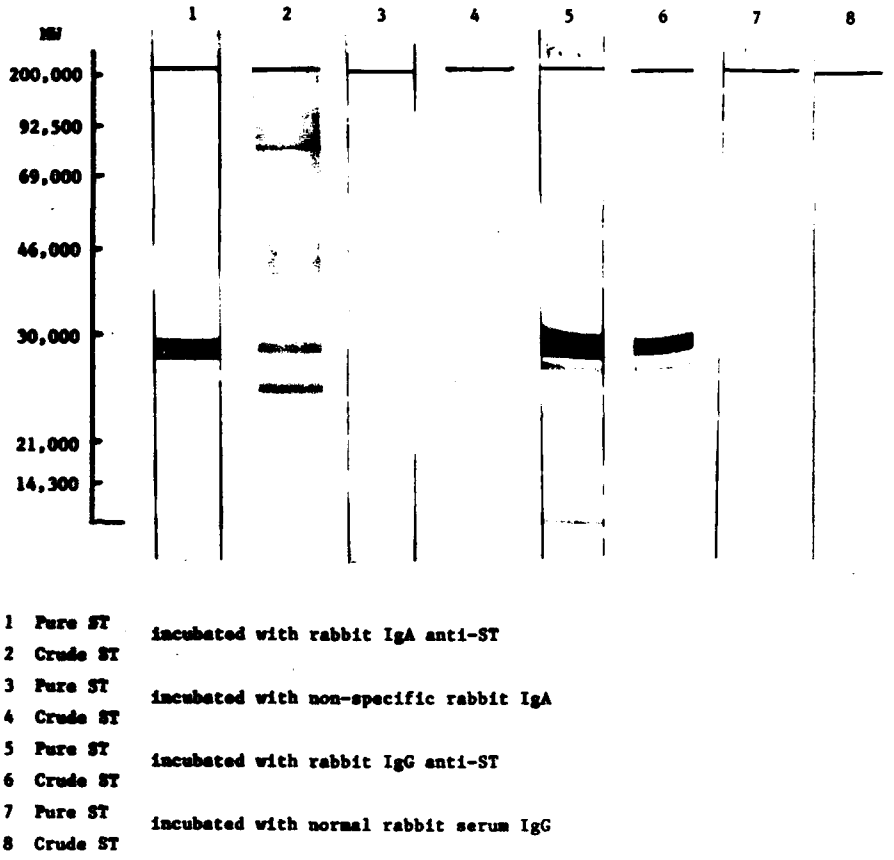

Figure 8. Western blot demonstrating specificities of IgA and IgG anti-Shiga toxin. IgA preparations from loop secretions. IgG preparations from rabbit serum.

D. Mouse Model for Mucosal Immunity to Shigella Antigens. While the chronically isolated ileal loop model in rabbits has allowed us to characterize many of the important variables involved in stimulating the mucosal memory response to *S. flexneri*, the outbred nature of the rabbit has limited the depth to which we could study the observed reactions. We are limited in terms of available reagents, genetic details of immune responsiveness and in the heterogeneity of the responses elicited. Furthermore, the lack of histocompatability prevents cross culture experiments with our available *in vitro* systems. Therefore, during this first year and a half, we have established a mouse model for examining the secretory IgA response to Shigella antigens. We have chosen Shiga toxin to study, as little is known about the mucosal immune response to this molecule and our preliminary studies in rabbits indicate that it may be a strong mucosal immunogen. Further, the antibody response to this antigen may protect against its toxic effects (see next section).

This first group of mice were given Shiga toxin according to the dosage schedule in Table 2. Mice #1,2, and 3 were given orogastrically 0.1ml of partially purified Shiga toxin (post-DEAE) in 0.4 ml saline and 0.5 ml of sodium bicarbonate. Mice # 4,5, and 6 were

given 0.5 ml Shiga toxin diluted in sodium bicarbonate. Unfortunately, due to the unfamiliarity with the technique, two animals died within the first week of the study.

Table 2. Dose Schedule for Oral Immunization of Mice with Crude Shiga Toxin Preparation.

Mouse Group	Dose Given[1]	Days Given[2]
Low Dose	0.1ml Shiga toxin	0, 7, 14
High Dose	0.5ml Shiga toxin	0, 7, 14

1. Preparation of Crude Shiga toxin as described in Methods mixed with .2mM Na HCO_3 and saline.
   For low dose group, .1ml Shiga toxin mixed with 0.5ml of 0.2mM $NaHCO_3$ and saline.
   For high dose group, 0.5ml Shiga toxin mixed with 0.5ml of 0.2mM $NaHCO_3$ and saline.
   Doses were divided and given 15 minutes apart.
2. Day of first immunization = Day 0.

An ELISA for specific IgG, IgA and IgM mouse antibodies to Shiga toxin was developed. Mouse IgG, IgA, and IgM (Sigma Chemical) were reconstituted in 2.5 ml of PBS to a concentration of 2mg/ml and electrophroresed on a high resolution agarose system. Each preparation contained only one major band (a small second band was seen in the IgM preparation). The mouse immunoglobulin preparations were used to coat microtiter plates for the ELISA. The cross-reactivity of these reagents as shown in figure 9 indicates that the anti-IgA and anti-IgG reagents are relatively specific when reacted with IgG or IgA, but that they cross-react somewhat with the IgM-coated wells. The anti-IgM reagent shows slight cross-reactivity with the IgA- and IgG-coated wells. To eliminate this cross reactivity of the anti-IgG and -IgA reagents with IgM, we added a 1/2000 dilution of IgM to both antisera.

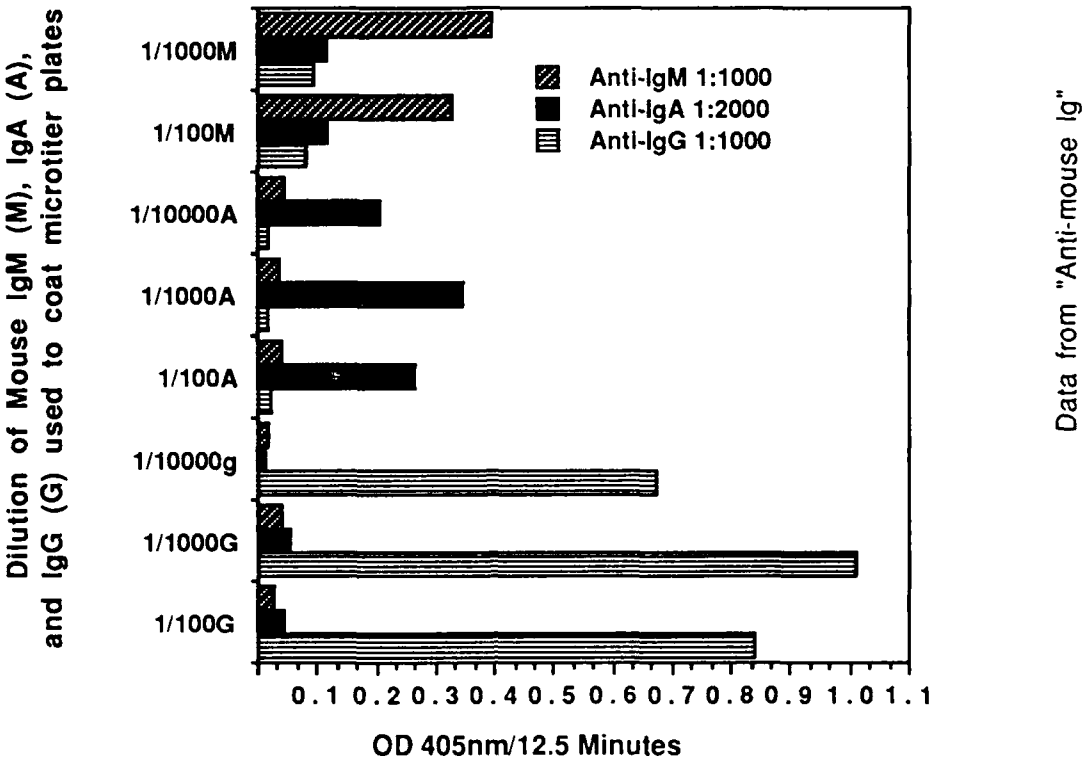

Figure 9. Development of isotype specific mouse immunoglobulin ELISA.

To determine the anti-ST content of the loop secretions, purified Shiga toxin preparations from Dr. J. Edward Brown were used to coat the microtiter wells. The concentration was 20ug/ml as used in the rabbit loop studies (see later). The secretions were diluted 1:2 in PTA and assayed by the ELISA as described in the Methods section. As shown in figure 10, both the low and high oral doses of crude Shiga toxin were able to stimulate a secretory IgA anti-Shiga toxin response. No IgG or IgM anti-Shiga toxin were detected in the secretions from these two animals.

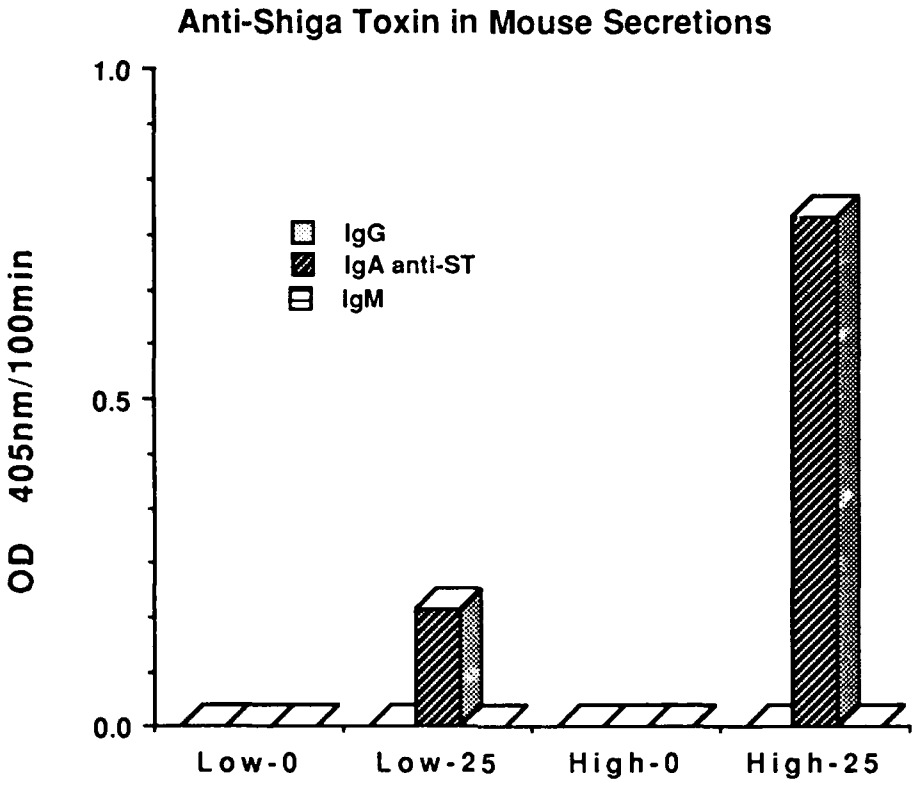

Figure 10. Anti-Shiga toxin response following oral immunization with low or high dose of the crude Shiga toxin preparations (see text).

At the same time, the serum from these mice diluted 1:40 in PTA had high titers of IgG anti-Shiga toxin activity with less IgA and almost no significant increase in IgM anti-Shiga toxin levels (figure 11).

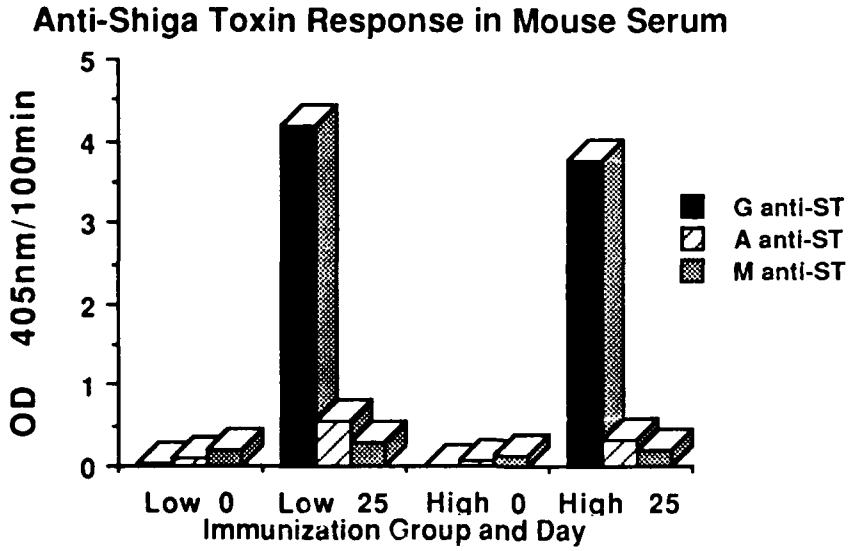

Figure 11. Anti-Shiga toxin response in serum from animals immunized orally with either low or high dose of crude Shiga toxin (see text).

The dichotomy between serum and secretory immunoglobulins in the results from these two groups of mice are the same as we and others have seen previously with such antigens as cholera toxin. Following oral immunization a strong local secretory IgA response is found with little or no IgG anti-Shiga toxin activity. At the same time, there is a high titer of IgG anti-Shiga toxin in the serum with only a small amount of IgA anti-Shiga toxin. Interestingly, while the secretions showed a higher level of activity of the IgA anti-Shiga toxin with the higher oral dose, the opposite effect was seen in the serum humoral immune response.

At the present time, however, these results only represent a few animals and considerably more work needs to be done before firm conclusions can be made. However, these extremely promising preliminary results indicate that the mouse model will be quite useful for studies of the mucosal immune response to Shiga toxin and possibly to other Shigella antigens.

E. Biologic Role of Secretory IgA Response to Shigella Antigens.

Two studies on the biologic effect of specific secretory IgA against shigella antigens have been completed at the present time. The first involves study of the ability of secretory IgA anti-Shigella LPS to interfere with the binding and uptake of live, virulent *S. flexneri* in an acutely ligated segment of intestine. The second demonstrates both *in vitro* and *in vivo* the ability of secretory IgA against Shiga toxin to interfere with the cytotoxic effects of that molecule.

1. Role of Secretory IgA to interfere with uptake of live *S. flexneri* by the intestinal epithelium. Based on the results of the studies conducted in our laboratory on the uptake of *S. flexneri* by follicle-associated epithelium, we inferred that the replication of these bacteria within these epithelial cells inexorably follows their entry into the cytoplasm. Therefore, preventing the initial entry, or decreasing the number of bacteria permitted to enter the epithelial cells should interfere with the damage to the surface epithelium. In a preliminary study, two rabbits were immunized with three oral doses of 10^8 live *S. flexneri* 2457-0 given once a week, and allowed to rest for two weeks. However, due to a difficulty in securing a consistently invasive strain (as shown by the Sereny test), we had to wait several weeks before conducting the protection studies. During this time, we gave three more booster doses to these rabbits. When a virulent strain of *S. flexneri* became available, we ligated loops of intestine in these two rabbits and in an unimmunized rabbit as a control. The control rabbit showed a dilated loop with bloody secretions after 18 hours. In marked contrast, there was no evidence of blood in the two immunized rabbits, although there was a watery fluid secretion in these loops. Histologically, the 18 hour loops from the control rabbit showed ulcerations over all the Peyer's patch dome areas with blood in the lumen. The immunized rabbits had rare ulcers with some dome regions completely intact. At the 90 minute time period, there was an 8-10 fold difference in the uptake of the bacteria by the surface epithelium in the immune and nonimmune animals.

With this preliminary information, we created five groups of rabbits (2 rabbits in each group) to determine the effect of prior immunization on the uptake of the shigella (Table 3)

Table 3. Dose Schedule for Shigella Uptake Study

Group	Antigen Given	Dose[1]	Days Given
I	*S. flexneri* 2457-O	10_8^8	0,7,14,35
II	*S. flexneri* M4243A1	10_8	0,7,14,35
III	*Shigella X16*	10_8	0,7,14,35
IV	*Culture Media*	10_8	0,7,14,35
V	*Heat-Killed S. flexneri*	10^8	0,7,14,35

1. Antigen given via orogastric tube.

On day 42, an acute loop study was conducted using 10^8 live *S. flexneri* M4243 injected directly into the isolated loops. The results shown in Table 4 indicate that there was no detectable protection in any of the groups. These results were contradictory to the preliminary studies. However, during the preliminary studies, we gave two more immunizing doses, and there was concern about the level of virulence of the strain of shigella used in the final assay. On evaluating the results, we identified two key factors which may have contributed to the lack of protection. First, the dose of virulent M4243 used (2×10^9) was very likely too high to allow demonstration of the protective ability . This many bacteria in the limited surface area of the ligated loop were probably able to overcome the secretory IgA which was present. Unfortunately, when we attempted to use fewer bacteria as a challenge, the amount of uptake of the shigella was too inconsistent to measure. Secondly, the periods examined (90 minutes and 18 hours) may have been far too long. These times were used because our previous study showed that uptake can readily be observed at these times using both ultrastructure and Giemsa staining (21). However, the initial attachment of the shigella occurs much sooner than this (likely prior to 30 minutes). Therefore, by looking at later time points, we have prejudiced the study in favor of eventual invasion by the shigella.

Table 4. Uptake of Shigella[1] in Rabbits Immunized Orally with Different Shigella Preparations

Antigen for Immunization	Bacteria[2] Uptake at 90 Minutes			
	Dome Regions		Villus Regions	
Culture Broth	2.79	(0.12)	0.62	(0.08)
Culture Broth	2.27	(0.04)	0.38	(0.03)
S. flexneri 2457-Q	8.35	(0.37)	1.33	(0.19)
S. flexneri 2457-Q	7.36	(0.53)	0.85	(0.24)
S. flexneri M4243A1	3.95	(0.09)	0.61	(0.06)
S. flexneri M4243A1	5.06	(1.04)	0.77	(0.28)
Shigella X16	1.61	(0.01)	0.29	(0.01)
Shigella X16	3.26	(0.35)	0.53	(0.10)
Heat-killed Shigella	4.27	(0.18)	0.91	(0.09)
Heat-killed Shigella	0.76	(0.17)	0.23	(0.08)

1. Animals immunized as described in Table 3. Invasive S. flexneri M4243 used to challenge the isolated loops (see text).
2. Uptake expressed as numbers of bacteria completely within host epithelial cells with standard error of means indicated.

In future studies, we believe the use of a RITARD system would allow us to use much more physiologic doses (from the human disease viewpoint) and would give us a better assessment of the role that secretory IgA plays in protection against uptake of shigella.

2. Role of Secretory IgA to Protect against the Cytotoxic Effects of Shiga Toxin. Our studies on the mucosal immune response to Shiga toxin and its functional significance were carried out in collaboration with Dr. J. Edward Brown. His laboratory provided the Shiga toxin preparations and performed the below listed HeLa cell assay. All the rabbit studies, immunizations and protection studies were performed in our laboratory.

Although the role of Shiga toxin in dysentery is unknown, it is cytotoxic to HeLa cells , causes fluid secretion in rabbit intestine and is lethal when injected parenterally to rabbits or mice (28,33).

For the present study, five rabbits were inoculated directly into chronically isolated ileal loops (see Methods section) on the day of surgery (day 0) and on days 7 and 14 postsurgery. They were given 0.5ml of crude shiga toxin preparation (see Methods section) in 4 ml of saline. Intestinal secretions were collected daily and blood samples were collected weekly. A new ELISA for shiga toxin was created for these studies. While the technical details of the assay are the same as detailed in the Methods section, a four point standard curve was assayed on each plate with the unknown samples. The reciprocal of the dilution giving an O.D. reading between the two lowest values on the standard curve was defined as the titer.

As shown in figure 12, a significant increase in the mean IgG anti-Shiga toxin titer over the day 0 value was detectable in serum by day 7 after the first intraloop immunization. This titer rose after the third dose on day 14 and did not change significantly through the end of the study period on day 30. In contrast to the high titer of IgG anti-Shiga toxin in the serum, only trivial amounts were detected in the loop secretions (figure 12). Thus only a small amount of serum IgG anti-Shiga toxin finds its way into the loop secretions (our previous studies have shown good stability of IgG in the chronically isolated ileal loops).

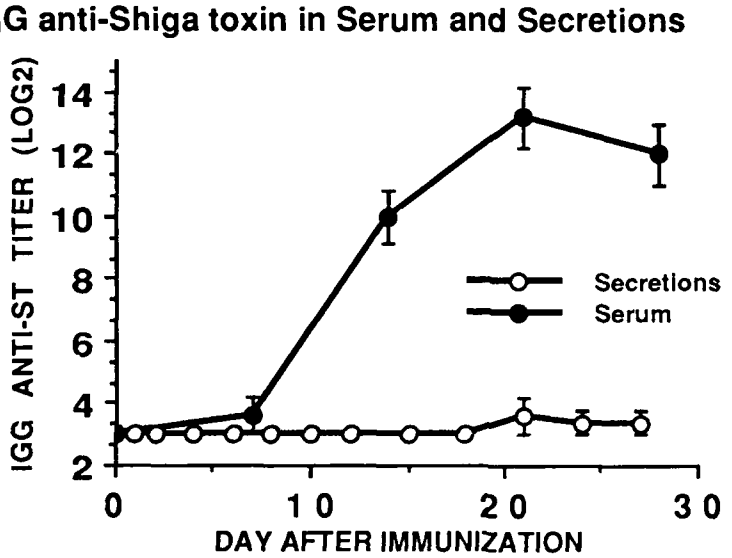

Figure 12. IgG anti-Shiga toxin in animals immunized orally with crude Shiga toxin.

The IgA anti-Shiga toxin titer in the serum of these rabbits was lower than the IgA titer in secretions (figure 12). A significant ($P<.01$) increase in IgA anti-Shiga toxin titer of the serum over the day 0 values was seen by day 14. In the loop secretions, as early as day 2, a weak but significant ($P<.05$) increase in the IgA anti-Shiga toxin titer was seen (figure 12). The content of IgA anti-Shiga toxin declined on the day after the third intraloop dose (day 14), but had another striking increase three days later. After this peak on day 18, the mean IgA anti-Shiga toxin titer slowly declined, although it never dropped below the level of activity seen after the second intraloop dose on day 7. It is possible that the slight decline in IgA titer seen the day following each booster immunization reflects the presence of free toxin in the loop which binds to the specific IgA. Alternatively, Shiga toxin may interfere with local antibody synthesis or secretion of IgA into the gut lumen.

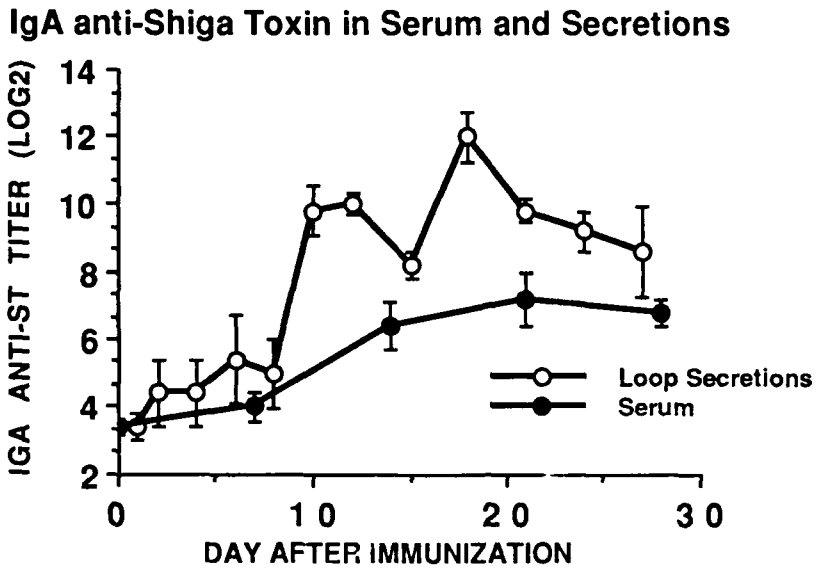

Figure 12. IgA anti-Shiga toxin in animals immunized orally with crude Shiga toxin.

To assess the *in vitro* Shiga toxin neutralizing activity of intestinal loop secretions, a HeLa cell assay was performed. For this assay, HeLa cell monolayers were grown in 96 well microtiter plates and a standard crude toxin lysate of *S. dysenteriae* was incubated with serial dilutions of loop fluids for 30 minutes at room temperature. This mixture was placed onto the HeLa cell monolayer and allowed to incubate overnight at room temperature. The monolayers were then stained with crystal violet and the O.D. 620nm was determined for each well. The dye remaining in each well correlates with the percentage of cells remaining adherent to the microtiter dishes (28). O.D. 620 nm of wells containing the standard toxin alone were averaged and that value plus two standard deviations was defined as the end point titer of loop fluids for neutralization of the cytotoxicity of the toxin preparation. All loop fluids which gave an O.D. 620nm in the assay greater than this value were scored as positive.

The Mean Shiga toxin neutralizing activity in the HeLa cell assay is depicted in figure 13. The curve in figure 13 shows the same basic triphasic response as the IgA anti-Shiga toxin in loop secretions from figure 12. The correlation coefficient of the mean IgA activity in secretions with the mean toxin neutralization titer was .928 while the correlation of the IgG level in secretions with the mean toxin neutralization titer was only .116.

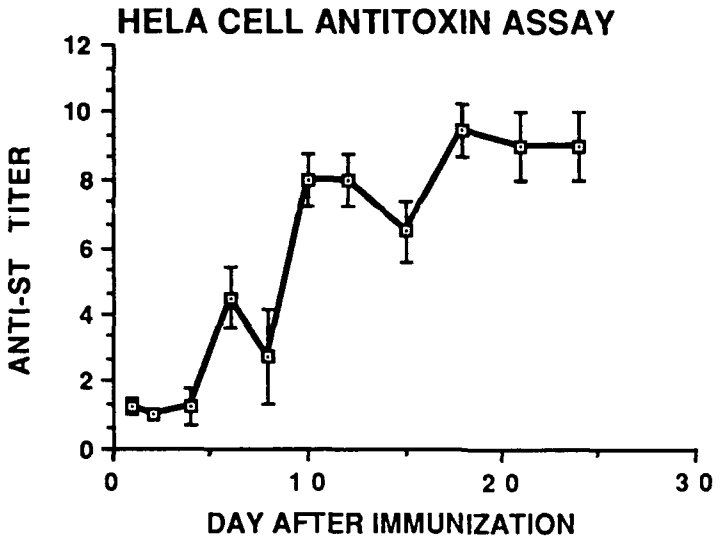

Figure 13. Anti-Shiga toxin activity *in vitro* of loop secretions from animals given oral immunization with crude Shiga toxin.

To assess the *in vivo* Shiga toxin neutralizing activity of intestinal loop secretions, an acute loop protection model was devised. Pooled loop secretions from rabbits with high titer IgA anti-Shiga toxin activity as determined by ELISA were diluted 1:2 in saline and mixed with an equal volume of a 1:256 dilution of crude toxin. This was injected into 5 cm isolated segments of ileum in unimmunized rabbits. This dose of toxin was chosen as it consistently elicited fluid accumulation when given to acutely ligated loops. As controls, toxin was mixed with secretions from nonimmune animals or saline and injected into other loops in the same rabbit. After 18 hours, the animals were sacrificed and the volume of fluid in each segment was measured.

Pooled loop secretions from animals immunized with Shiga toxin reduced toxin-induced fluid accumulation in the acutely ligated rabbit intestine. Secretions with no detectable IgA or IgG-anti-Shiga toxin by ELISA had no inhibitory effect on the Shiga toxin-induced fluid production by rabbit intestine (figure 14). The heterogeneity shown by the standard errors of the means reflects the differential response of the genetically diverse outbred rabbits used in these studies. Even with this degree of heterogeneity, the difference between the fluid production in loops protected with immune secretions and those given nonimmune secretions was highly significant ($P<.01$).

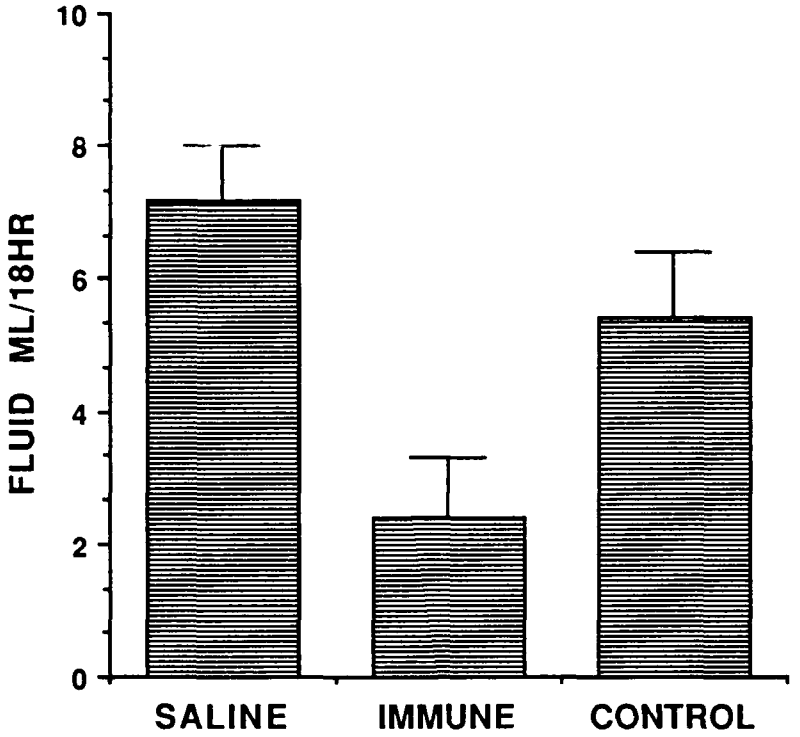

Figure 14. Anti-Shiga toxin activity *in vivo* of loop secretions from animals given oral immunization with crude Shiga toxin.

These findings indicate that a strong secretory IgA mucosal immune response can be elicited to Shiga toxin and suggest that such antibodies could interfere with the toxic effects both *in vitro* and *in vivo*.

CONCLUSIONS

These studies demonstrate several major characteristics of the local secretory IgA response to shigella antigens. First, a major route of uptake of the intact shigella is by the M cells which lie in the follicle-associated epithelium overlying lymphoid follicles throughout the gastrointestinal tract. When the *S. flenxeri* taken up are virulent, they proliferate within these cells eventually producing ulceration at these sites. The presence of many such follicles in the colon and the terminal ileum may explain the frequency of focal ulcerations at these sites in clinical dysentery. Ideally, one would wish to interfere with the initial adherence of the shigella to the surface epithelium to prevent clinical disease. Since our previous studies have shown a strong secretory IgA memory response in intestinal secretions following oral immunization with live, attenuated strains, we sought to use intact rabbits as a model system to evaluate the ability of secretory IgA to interfere with the uptake and ulcerations which occur. For these studies, we performed preliminary investigations with rabbits given several oral doses of live, attenuated strains of shigella. However, in order to obtain reproducible uptake and ulcerations, large doses of shigella were required. This, of course, contrasts with the human situation were only small numbers of shigella can produce clinical dysenetery. Therefore, we were not able to demonstrate protection

with the vaccinated animals. During the next period, we will be using the RITARD model to determine the minimal effective dose of shigella. When we obtain pathologic effects with doses more approximating the human situation, we will repeat the protection trial studies.

The cellular basis for the mucosal memory response is a key parameter to establish in order to expedite future trials of potential vaccine strains and to provide an understanding of the mechanisms involved in stimulating mucosal memory to enteropathogens. These studies are proceeding ahead of schedule. We have established cell cultures from Peyer's patches, mesenteric lymph nodes, spleen and peripheral blood from animals given various immunization regimens with attenuated strains of *S. flexneri*. From these studies, it is clear that by the third day following oral rechallenge, memory B cells are present within the Peyer's patches and mesenteric lymph nodes. In unimmunized animals, virtually no antigen-specific response is found on these days after oral challenge. Further, by this time, the cells are already committed to synthesize the IgA isotype. Studies during the next period will examine the T cell regulation of this response (as it is known from other studies that the IgA response is highly T cell dependent).

We have examined the heterogeneity of the secretory IgA response against Shiga toxin. These studies have demonstrated for the first time that a strong secretory IgA response can be elicited to Shiga toxin and that this response can protect both *in vivo* and *in vitro* against the toxic effects of that molecule. These findings have also established Shiga toxin as a potential mucosal adjuvant which may be used to augment the mucosal immune response to other antigens. Only cholera toxin has given such a strong secretory IgA response in our rabbit model system.

A mouse model system to evaluate the mucosal immune response to shigella antigens has been established. The lavage system in mice has been refined to the point where we can expect 80-90% of animals to survive experiments lasting for one month. The initial data indicate that a strong secretory IgA response to Shiga toxin is elicited and that the level of the response seems to be dose dependent. We will continue to use the mouse model to evaluate the ability of Shiga toxin to act as a mucosal adjuvant. Further, by establishing the mouse model against shigella antigens, many monoclonal reagents and inbred strains are now available which allows us to dissect more precisely the cellular basis of the secretory immune response to shigella antigens.

In summary, these studies have provided much needed information on how to stimulate the mucosal memory response to shigella antigens. While this work has obvious practical implications to shigella many of the findings, especially the possible role of Shiga toxin as a mucosal adjuvant, have potential application to other enteropathogenic infections is also provides basic details about the secretory IgA memory response and its functional significance. Information about the cellular basis of the mucosal memory response will allow us to test potential vaccine strains against a wide variety of infectious agents in a fraction of the time and expense previously required by the reliable, but slow and costly Thiry-Vella loop technique. During the next year and a half, we will complete the evaluation of the cellular basis for the memory response in rabbits, will establish in detail the mouse model for mucosal immunity and will determine whether Shiga toxin can serve as a mucosal adjuvant to enhance the secretory IgA response against other enteropathogens.

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APPENDIX

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Role of M Cells in Initial Antigen Uptake and in Ulcer Formation in the Rabbit Intestinal Loop Model of Shigellosis

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Strains of *Shigella flexneri* with different invasive and pathogenic potentials were inoculated into the intestinal lumen of acutely ligated loops in nonimmune rabbits. After 90 min, tissues processed for ultrastructural as well as light microscopy showed that the bacilli were phagocytosed by M cells over lymphoid follicles of Peyer's patches and carried in vacuoles into the epithelium. Nonpathogenic as well as pathogenic strains were readily taken up regardless of the presence of the 140-megadalton virulence plasmid. More virulent than avirulent shigellae were found in M cells at 90 min, reflecting replication or preferential uptake of the virulent strains. Heat-killed shigellae of the virulent strain were taken up by M cells to the same degree as the avirulent strains. Incubation of the bacteria for 18 h resulted in surface ulceration which was limited to epithelium overlying lymphoid follicles (M cell areas) in acute loops exposed to the virulent shigellae. Villus epithelium adjacent to the ulcerated follicular domes was intact, although there was mucus depletion. In the present study, we found that pathogenic shigellae appear to replicate in the M cells, escape from the phagocytic vesicles, and thereby initiate the ulcerations in this experimental model of dysentery. While initial antigen processing in the gut for a mucosal immune response may require uptake of luminal microorganisms by M cells, this may pose a threat under some circumstances.

Shigellosis is an acute bacterial enteritis, which in humans involves principally the colon. Often both the ileum and the colon are affected with focal mucosal ulceration. The classical picture is abdominal cramps, colicky pains, and an early diarrheal phase (likely due to the action of *Shiga* toxin) characterized by the passage of voluminous watery stools, followed hours or days later by a dysentery phase. In the latter, *Shigella flexneri* bacilli invade the surface epithelium, multiply, migrate laterally from cell to cell, and enter the lamina propria (16). Finally, superficial ulceration and bleeding occur. As a result, patients with dysentery pass frequent small-volume stools containing blood, mucus, and pus in addition to the previously mentioned symptoms (8, 16, 23, 25).

Our laboratory has used a chronically isolated ileal loop model in rabbits to follow, in intestinal secretions, the secretory immunoglobulin A (IgA) response to immunization with *Shigella flexneri*. In those studies, either oral or intraloop immunization with invasive or noninvasive *S. flexneri* consistently initiated a secretory IgA response to *Shigella* lipopolysaccharide (LPS) (9–11). Furthermore, invasive *Shigella* strains containing the 140-megadalton virulence plasmid were no more effective at achieving a strong secretory IgA response to *Shigella* LPS than were noninvasive strains. A noninvasive strain, *S. flexneri* M4243A$_1$, which lacks the 140-megadalton virulence plasmid, given orally was as effective at eliciting a mucosal memory response to *Shigella* LPS as was locally invasive *Shigella* strain X16 (containing the virulence plasmid) (12). In the present studies, we used an acute ileal loop model in rabbits to determine whether the ability of noninvasive strains to stimulate a mucosal immune response depends on their phagocytosis by specialized lymphoid follicle-associated epithelial cells (M cells) previously described to be responsible for the initial processing of luminal macromolecules and microorganisms (1, 5, 7, 17–20, 22, 27, 28). In addition to providing information by sampling of intact shigellae, the present findings indicate that the initial ileal ulcerations in this model occur over intestinal lymphoid follicles.

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MATERIALS AND METHODS

Bacterial strains used. Strains of *S. flexneri* used were originally provided from the laboratory of Samuel B. Formal at the Walter Reed Army Institute of Research. *S. flexneri* M4243 is a virulent strain which can invade the epithelium, proliferate, and cause superficial ulceration. It contains the 140-megadalton virulence plasmid and produces a positive Sereny test (2). *Shigella* strain X16 is a hybrid of *S. flexneri* and *Escherichia coli*. It also contains the 140-megadalton virulence plasmid and is able to invade the surface epithelium. However, it does not persist after invasion, does not replicate, causes no ulceration (3), and does not give a positive Sereny test. *S. flexneri* 2457-0 is a noninvasive strain, although it retains the 140-megadalton virulence plasmid. It neither causes ulcer formation in rabbit intestinal mucosa nor gives a positive Sereny test (4). *S. flexneri* M4243A$_1$ is a noninvasive strain which lacks the virulence plasmid (24). Predictably, this strain neither causes ulcer formation nor gives a positive Sereny test. To see the difference in uptake between live and killed bacteria, we studied the uptake of heat-killed M4243. The overnight bacterial culture was heated in boiling water for 10 min. The heat-killed M4243 culture did not grow when streaked on a MacConkey plate.

Preparation of acute ileal loops. For each strain of *Shigella* studied (Table 1), three New Zealand White rabbits weighing about 3 kg each were anesthetized with Xylazine and Ketamine. A midline abdominal incision was made, and the ileum was identified. Then, 8- to 10-cm loops containing a grossly identifiable Peyer's patch were constructed by first tying off the proximal end with 4.0 silk, flushing the intestinal contents distally with sterile saline, and then tying off the distal end with 4.0 silk. The blood supply to the loop was kept intact.

TABLE 1. Rabbits studied and main characteristics of each bacterial strain

Rabbit group	Bacterial strain	Virulence plasmid	Mucosal ulceration	Mucosal uptake	Sereny test
1	M4243	+	+	+	+
2	X-16	+		+	
3	2457-0	+		+	
4	M4243A	−		+	
5	Heat-killed M4243	+	−	+	

An overnight culture (2 ml, 2×10^9 bacteria) in brain heart infusion broth (Becton Dickinson and Co., Cockeysville, Md.) was injected into the closed loops, which were returned to the abdominal cavity. Peyer's patches were arranged in a dependent position, and the loops were gently massaged every 10 to 15 min to ensure even exposure of the Peyer's patches to the shigellae. The loops were allowed to incubate for 30 or 90 min. For studies on pathogenicity of each strain, an 18-h incubation was used. After the specified incubation period, the loop was excised intact and opened along the mesenteric border, and the Peyer's patch with a surrounding rim of the intestinal wall was sampled. One piece of the sample was fixed in glutaraldehyde-formaldehyde for electron microscopy, and the rest was snap-frozen in liquid nitrogen for histologic assessment of bacterial uptake.

Electron microscopy. Tissues were minced to approximately 1 mm^3 and fixed in 3% glutaraldehyde–formaldehyde in 0.1 M cacodylate buffer, pH 7.3 (Tousimis Corporation, Rockville, Md.). The samples were postfixed in 2% osmium tetroxide. After being stained en bloc with 2% uranyl acetate, tissues were dehydrated in alcohol and embedded in Epon. Sections (1-μm thick) were cut, stained with toluidine blue, and examined. Areas of follicle-associated epithelium (FAE) containing shigellae were chosen. Thin sections approximately 80 nm thick were then cut from the selected areas on a Porter-Blum MT-2 ultramicrotome. These sections were stained with lead citrate and examined with a Zeiss 109 transmission electron microscope. Photomicrographs were taken of the characteristic rod-shaped bacteria in the FAE.

Light microscopy. Frozen sections (6-μm thick) were obtained along the longitudinal axis of the bowel through the Peyer's patch and adjacent intestine. The sections were immediately fixed in 100% ethanol and stained with Giemsa. These sections were examined with an oil immersion lens.

The surface epithelium was divided into two regions for this study. The dome area over the Peyer's patch, which is enriched with M cells, was the first region. Few goblet cells are found in this epithelium. Wandering lymphocytes and macrophages and connective tissues underlie the FAE (18). The second area examined consisted of villi outside the Peyer's patches. These structures contain primarily absorptive columnar cells and numerous goblet cells. An image analyzer (Bioquant, Biometrics, Nashville, Tenn.) with an IBM PC was used to measure the actual length in millimeters of the lining epithelium over the villi and over the dome regions of Peyer's patches. The mean of actual length of 100 areas for dome and villus areas from representative rabbits was calculated. The average length of dome epithelium was 1.26 ± 0.03 mm (standard error) and of villus epithelium was 1.36 ± 0.04 mm.

We examined five coded slides per rabbit and counted bacteria taken up over each dome area and over 10 randomly selected villi. These numbers were presented as bacteria per millimeter of surface epithelium of villi or domes. The mean and standard error of the mean were then calculated from

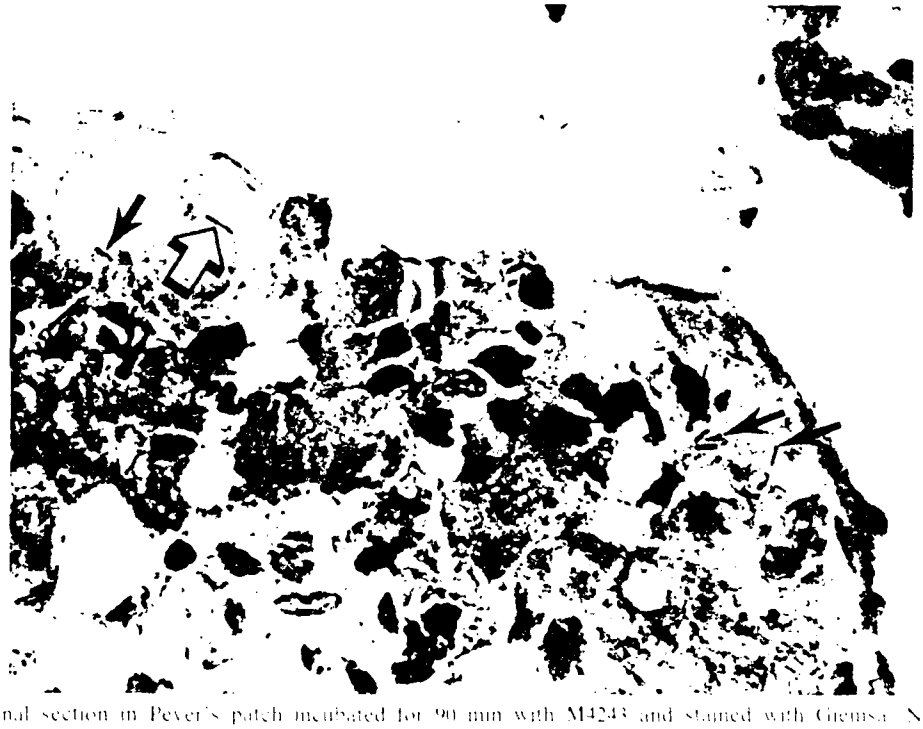

FIG. 1. Longitudinal section in Peyer's patch incubated for 90 min with M4243 and stained with Giemsa. Note the characteristic rod-shaped bacilli incubated for 90 min and taken up by epithelium over dome areas (arrows). The open arrow points to a bacterium undergoing binary fission. Bacteria may be seen in cross sections or oblique sections. Magnification, ×825.

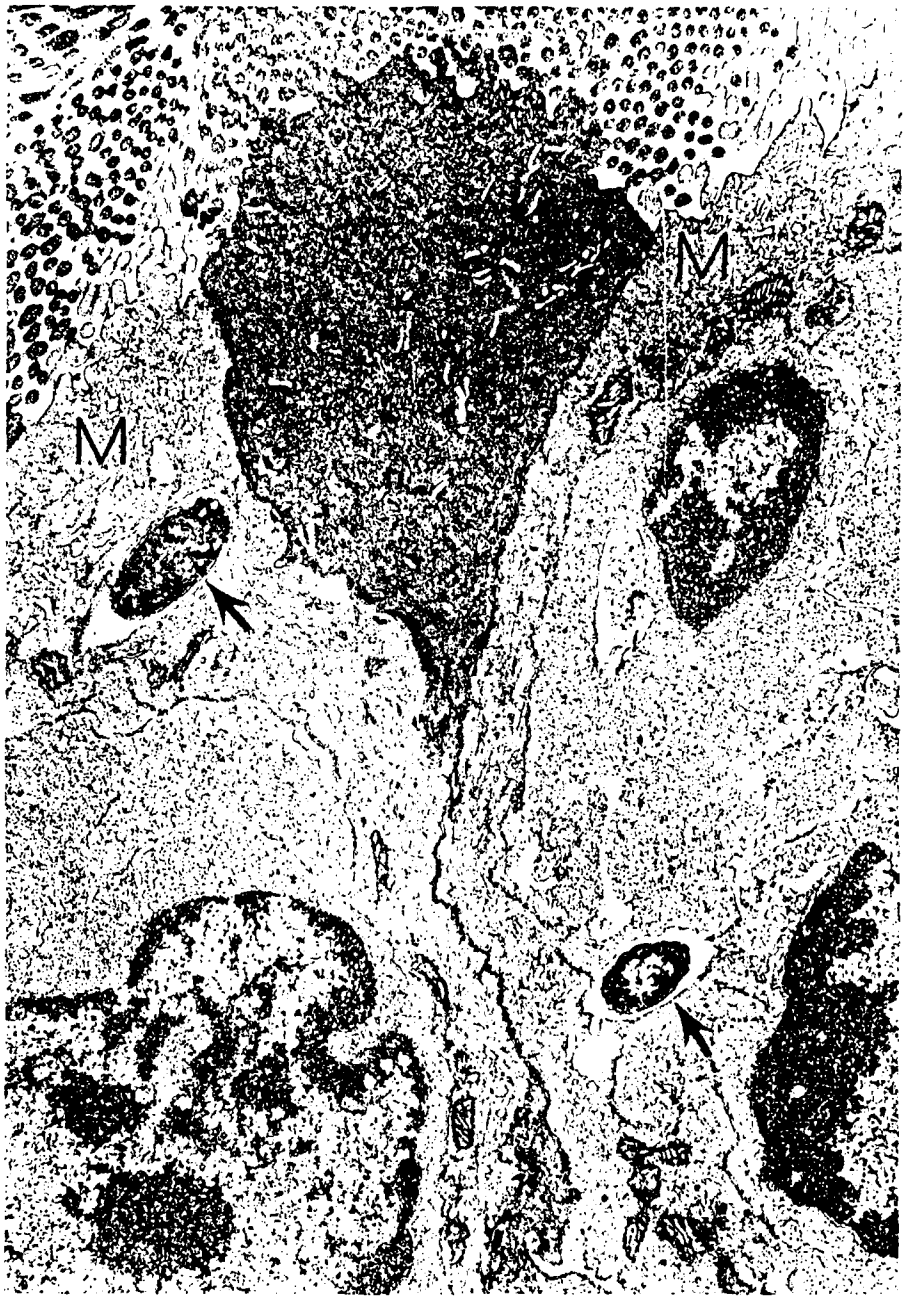

FIG. 2. Transmission electron micrograph of M cells containing shigellae packaged in vacuoles (arrows). Magnification, ×13,000.

150 observations over villus epithelium or about 120 to 130 observations over FAE for each group of rabbits studied.

Statistical analysis. Results were compared by Student's *t* test on RS/1 software.

RESULTS

Uptake of shigellae at 30 min. At the 30-min time point, there was almost no demonstrable uptake of *S. flexneri* by either the FAE or the villus epithelium. No damage or evidence of inflammation was seen at this early time. A rare shigella was seen in the apical portion of the epithelium over the lymphoid follicles in the M4243 strain group.

Uptake of shigellae at 90 min. All four *Shigella* strains were readily detected in the dome epithelium by 90 min. We required that the entire shigellae be within the cytoplasm to be counted (Fig. 1). Bacteria which were adherent to the surface epithelium but not clearly present within the cytoplasm were not counted. As shown by ultrastructural studies, the bacteria were taken up by the M cells in vesicles. All the bacteria seen at the 90-min point by ultrastructural studies were contained within membrane-lined vesicles (Fig. 2). The pathogenic strain *S. flexneri* M4243 had significantly greater numbers of bacteria within the dome epithelium than did the three nonpathogenic strains ($P < 0.01$) (Fig. 3). In several areas, the M4243 strain showed clustering of bacteria, some of which were undergoing binary fission (Fig. 1). The nonpathogenic strains were taken up with equal efficiency regardless of their invasive abilities or the presence of

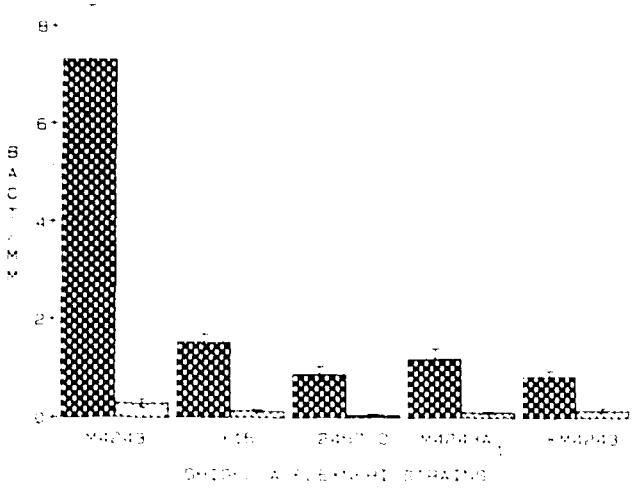

FIG. 3. Uptake of different *Shigella* strains by 1 mm of FAE (checkered columns) versus 1 mm of villus epithelium (hatched columns) after 90 min of incubation. KM4243 is heat-killed M4243. Data are expressed as mean ± SEM. BACT/MM, Bacteria per millimeter.

the 140-megadalton virulence plasmid (Fig. 3). Interestingly, heat-killed pathogenic M4243 were taken up by M cells as well as live avirulent bacteria (Fig. 3).

All strains examined had relatively few shigellae within the villus epithelium (uptake by dome epithelium was at least 10-fold greater than that by villus epithelium for each strain). The uptake of pathogenic M4243 strain by villus epithelium was significantly greater ($P < 0.01$) than uptake of the nonpathogenic strains.

Incubation for 18 h. Mucoid fluid accumulation was seen in the acute loops incubated with every *Shigella* strain studied. With the pathogenic *S. flexneri* M4243, the fluid was bloody, and the wall of the loop was noticeably edematous, very friable, and sometimes coated with clotted blood. This was not the case with any of the other strains studied except the X16 strain, where there was some hemorrhage in the lumen and corresponding small focal ulcerations over some dome regions. Corresponding to the gross observations, microscopic examination revealed profound mucosal ulceration exclusively with the *S. flexneri* M4243 strain (Fig. 4). Many microorganisms were seen in the exudate over the ulcer and within the lymphoid follicle tissue. The surface was hemorrhagic, with marked acute inflammation throughout the lamina propria. Ulcerations were present almost exclusively in the dome regions over the Peyer's patches (Fig. 4), but occasionally extended to the bases of adjacent villi and caused their avulsion. These sloughed villi left flat hemorrhagic ulcers. Although there was mucosal damage in the attached or sloughed villi (edema, mucus depletion), the surface epithelium was in general intact (not ulcerated). In contrast, the nonpathogenic shigellae were not found within the surface epithelium at this time point. The three nonpathogenic strains showed no ulceration after the 18-h incubation.

DISCUSSION

Rabbit ileum was chosen as the site for these studies because it is enriched in Peyer's patches, which are a major site for uptake of luminal antigens by the intestine (1). In humans the ileum is the second most frequent site, after the colon, of mucosal ulceration in bacillary dysentery (25).

In the present study, we found that both pathogenic and nonpathogenic *Shigella* strains were taken up by the specialized M cells in the FAE which overlies dome areas in Peyer's patches and isolated lymphoid follicles in the gastrointestinal tract. M cells have been shown by other workers to be involved in pinocytosis of macromolecules as well as in sampling of luminal microorganisms (1, 5, 7, 17–20, 22, 27, 28). Consequently, the M cells are thought to play an important role in the initial uptake of antigens to stimulate the mucosal immune response.

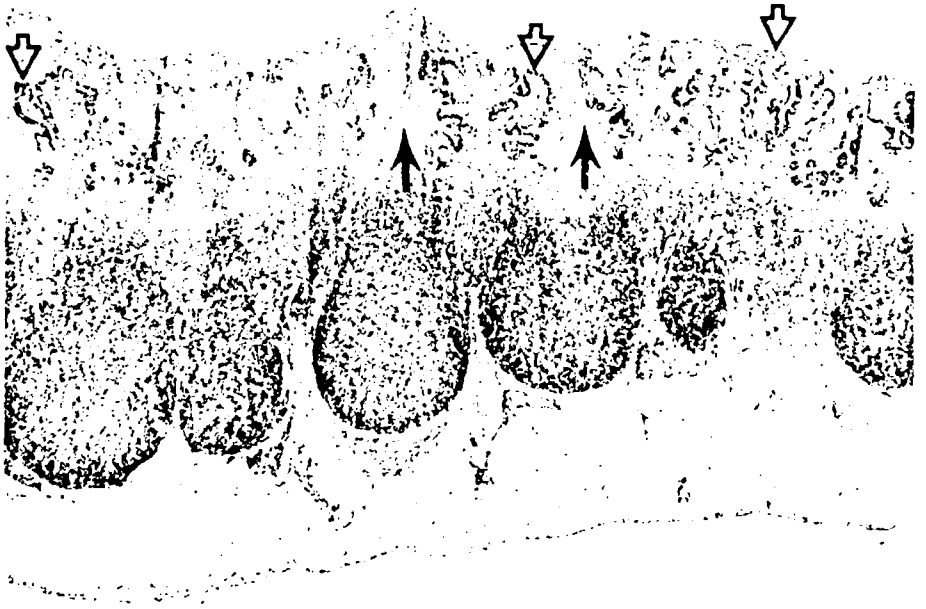

FIG. 4. Longitudinal section in rabbit Peyer's patch after incubation for 18 h with pathogenic M4243. Note the profound mucosal ulceration replacing the epithelium over the dome areas (solid arrows) and that although the villus epithelium is abnormal, it is intact (open arrows). Magnification, ×41.25.

Whereas the four live strains and one heat-killed strain of *S. flexneri* studied have dramatically different invasive and virulence potentials, all five preparations have been found to elicit significant mucosal immune responses in our previous studies, where direct intestinal stimulation was given in chronically isolated ileal (Thiry-Vella) loops (9–12). When the three nonpathogenic strains were administered orally, all three could prime the rabbits for a memory mucosal response regardless of their ability to invade the surface epithelium or of the presence of the 140-megadalton virulence plasmid (11, 13). We predicted that all three should be sampled with equal efficiency by the surface epithelial M cells. The findings in the present study are consistent with this hypothesis. The killed preparation, however, was not able to elicit a memory mucosal immune response even when large doses of antigen were given orally (13). Since Owen et al. had shown previously that live but not killed *Vibrio cholerae* were taken up by M cells (20), we had hypothesized that the killed shigellae did not elicit a mucosal memory response due to ineffective sampling by the M cells. The present studies clearly demonstrate that this earlier hypothesis was wrong. The killed shigellae were taken up to the same extent by M cells as were the avirulent strains which can elicit a vigorous mucosal memory response. The reason that killed shigellae were unable to elicit a memory mucosal immune response is not known at present. Perhaps the process of bacterial division is necessary to prolong the existence of these antigens in the lumen; alternatively, memory cells may be stimulated by epitopes which were destroyed by the heat treatment.

Although there was no significant difference in the number of shigellae within the epithelium for the live, avirulent strains or the heat-killed M4243 strain, there was a significant increase in the number of bacteria found in dome epithelium by 90 min with the pathogenic *S. flexneri* M4243. This may reflect either preferential uptake of the virulent strain by the FAE or intracellular proliferation after the initial uptake. We believe that the latter mechanism is best supported by the results of the present study. First, some bacteria were undergoing binary fission in addition to being present in scattered foci or groups (after 90 min of incubation). Second, after 18 h of incubation, enormous numbers of shigellae were in the tissues and the follicular surface epithelium was completely destroyed only by the pathogenic strain. Third, the four- to eightfold difference between the numbers of bacteria in loops incubated with virulent versus avirulent strains corresponds well to the intracellular rate of growth of shigellae in HeLa cell cultures (15). On the other hand, avirulent shigellae appeared as scattered, rare, and individual bacteria, not in clusters.

It is notable that when the pathogenic bacteria were allowed to incubate for 18 h in the acute loops, ulcerations preferentially occurred over the dome regions of the Peyer's patches. While the entire epithelium was damaged, only the areas over the dome regions of the Peyer's patches were completely denuded (Fig. 4). Ulcerative lesions extended to the bases of neighboring villi and crypts. Many villi appeared, solely or as a cluster, detached from the intestinal wall and free in the lumen. Microscopic examination of the ulcer revealed fibrinopurulent exudate matted together with sloughed epithelium and myriads of bacteria. Ulcerated areas were separated by zones of intact epithelium. The lesions in these ileal loops closely resemble those described for shigellosis in humans (21, 25). This suggests that in addition to being a site for antigen sampling and stimulation of the mucosal immune response, M cells may serve as the preferential portal of entry for pathogenic microorganisms such as *S. flexneri*. Indeed, others have proposed the M cell as a portal of entry for intestinal pathogens (5, 7, 14, 17, 20, 27) and even the human immunodeficiency virus (26). The presence of abundant M cells in the FAE is the main characteristic that differentiates this specific epithelium from that found throughout the gastrointestinal tract. These findings support our hypothesis that FAE is both the site of antigen sampling and the site of disease propagation and ulcer initiation in shigellosis.

It will be important to determine whether the uptake of virulent shigellae by the epithelium overlying lymphoid follicles can be prevented by the production of antigen-specific secretory IgA. Mucosal immunity has been shown to prevent the pathologic effect of *V. cholera* and cholera toxin (20). Since nonpathogenic strains are effective immunogens for developing a secretory IgA response to *S. flexneri* LPS, future studies with live nonpathogenic mucosal vaccines may be useful in the prevention of enteropathogenic diseases by interference with uptake of the causative microorganisms.

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Intestinal Mucosal Immune Defense Mechanisms

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The intestinal mucosal immune defense mechanisms involve both humoral and cellular immunity. The prominence of suppressor/cytotoxic T lymphocytes in the epithelial layer suggests that these interepithelial lymphocytes play a role in defense against infections within this layer. Secretory IgA is overwhelmingly the major humoral immune response along the gastrointestinal tract and along other mucosal surfaces (respiratory tract, mammary glands, salivary glands, and lacrimal glands). While the functions of secretory IgA are incompletely understood, it is clear that it prevents attachment of microorganisms and toxins (cholera toxin, shiga toxin, etc.) to the surface epithelial cells. Furthermore, secretory IgA may collaborate with eosinophils or killer lymphocytes to mediate cytotoxic reactions against enteropathogens. By learning more about the mucosal immune response, we should be able to understand the relationship between the lamina propria plasmacytosis in inflammatory bowel disease and the increased number of interepithelial lymphocytes that we see in gluten-sensitive enteropathy and the underlying pathogenic mechanisms.

Key Words: Secretory IgA—Mucosal immunity—Vaccine—Immunosuppression—Gut-associated lymphoid tissue—Peyer's patch—Isolated lymphoid follicle—M cell—AIDS.

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The discovery that IgA is the main antibody on mucosal surfaces provided the key to beginning definitive work to understand the biology of the mucosal immune system (1). While many tissues (bronchial mucosa, mammary glands, conjunctiva, genitourinary tract, etc.) are involved in mucosal immunity, the gastrointestinal tract is the major site of antigenic stimulation and immune response for secretory IgA (2).

It is well known that parenteral administration of antigenic material results in the formation of a systemic immune response directed to that antigen. Depending on the antigen, its dose, and the genetic capabilities of the animal, a humoral or cellular immune response will result. Yet, despite the fact that the plasma cells in the lamina propria of the gut constitute the largest collection of plasma cells in the body, it is not well known that antigenic material that passes along the gastrointestinal tract also elicits an immune response. The response is not, however, usually manifest by a strong systemic (IgG) immunity to the antigen; rather there is a local secretory IgA response with some, as yet poorly defined, local cellular component. Further, a dichotomy exists between systemic and mucosal humoral immunity, such that stimulation of one often results in suppression of the other. Current experimental vaccine trials are attempting to improve mucosal immunity to pathogens and their toxic products which gain access through mucosal surfaces (mainly gastrointestinal and respiratory) (3).

ANTIGEN PROCESSING IN THE GUT

Initial processing of antigens for stimulating a mucosal immune response is thought to occur in one of several major structures of the gut-associated lymphoid tissue (GALT). These consist of Peyer's patches, isolated lymphoid follicles, the appendix, and mesenteric lymph nodes. These structures have in common the presence of lymphocytes

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that have different functional capabilities from lymphocytes located in peripheral lymph nodes.

Primordial lymphoid structures are first detected along the intestine about halfway through gestation. At birth, Peyer's patches have the greatest density of any proliferating lymphoid cells in the body (4). After birth, there is a marked increase in number of Peyer's patches reflecting the initial response of the host immune system to the wide variety of environmental antigens that pass through the gastrointestinal tract. That this increase is related largely to microorganisms is supported by the finding that germ-free animals have Peyer's patches that are quite small, but that enlarge when the animals are exposed to microorganisms. Further, the number of lamina propria plasma cells is also known to be affected by antibiotic administration in animals, a factor most likely related to decrease of intestinal flora (5).

The epithelium overlying gut lymphoid tissues contains a population of epithelial cells that are specialized in their ability to take up luminal antigens and transport them to underlying lymphocytes. These cells are termed *M cells*, which is meant to indicate that they form a thin membranous barrier between the lumen and the underlying lymphoid populations (6). M cells have been detected over Peyer's patches, isolated lymphoid follicles, and the appendix (7–9). Further, the specialized epithelium overlying lymphoid follicles has few goblet cells and an abundance of interepithelial lymphocytes (Fig. 1). Mucus in goblet cells would likely interfere with initial attachment to M cells of microorganisms and other antigenic material in the gut lumen. M cells can sample small proteins and complex microorganisms (6,8). This sampling of antigen might not always be beneficial to the host. Some viruses and pathogenic bacteria are known to thrive after ingestion by an M cell (10) (Fig. 2). Along these lines, it has been suggested that M cells in rectal follicles may serve as the portal of entry for the AIDS virus during anal intercourse (11).

Another cell that may collect luminal microorganisms is the Paneth cell. Much less is known about this cell than about the M cell. However, it is clear that the Paneth cell hyperplasia, which occurs in blind loop syndrome and in models using isolated intestinal loops, is related to intestinal bacterial overgrowth (12). Furthermore, these cells have been shown to be phagocytic both in vivo and in vitro, suggesting that they respond to microorganisms in the gut (13). Paneth cells are also known to contain lysozyme, which probably serves an antibacterial function (13). The lack of a close relationship between Paneth cells and lymphoid follicles makes a protective role more likely than an antigen-processing role for these cells.

After mucosal antigens are sampled by M cells, the antigen is transferred to underlying lymphoid tissues where it is processed to stimulate the mucosal immune response. GALT contains several key populations of lymphocytes which differ in functional capabilities from their counterparts in peripheral lymph nodes. Precursor B lymphocytes with surface IgM or IgD predominate. They will preferentially mature to become IgA-secreting plasma cells (14). While some of the capabilities of these IgA precursor B lymphocytes may be in-

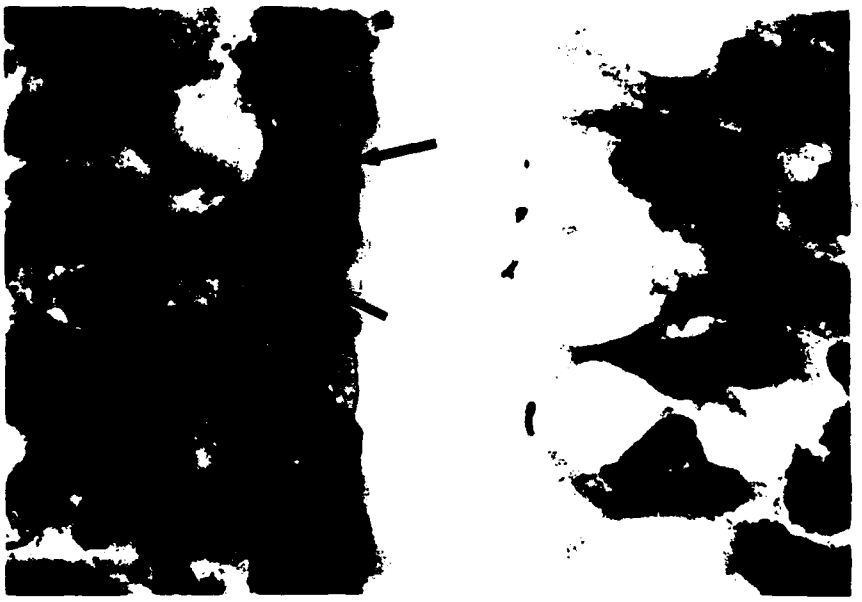

FIG. 1. This photomicrograph depicts the follicle-associated epithelium over the dome area of an ileal Peyer's patch in a rabbit. The animal had ingested colloidal carbon for several hours prior to obtaining this section. Note the attachment of the carbon to the surface epithelium (arrows) and uptake by the specialized epithelium (arrows). Relatively little carbon is adherent to the adjacent villus epithelium (at right).

FIG. 2. This electron micrograph shows uptake of *Shigella flexneri* by an M cell over a Peyer's patch. Such uptake of pathogens by M cells may serve as a portal for entry of some infectious agents. (lead citrate, uranyl acetate, ×7,300).

herent in their genetic programming, their maturation is profoundly influenced by T lymphocytes present within GALT.

Several immunoregulatory T lymphocytes have been described in GALT. By growing clones of T lymphocytes from Peyer's patches and the spleen, Kawanishi et al. were able to demonstrate the existence of a population of T cells that would alter the surface immunoglobulin expression of the Peyer's patch B lymphocytes form IgM to IgA (Table 1)

TABLE 1. *Effect of switch T cell clone on Peyer's patch B cell surface isotype expression*

Surface Ig	PP B cells (%)	PP B cells plus switch T clone (%)
IgM	31.6	31.4
IgG	16.0	11.7
IgA	4.5	43.6

Data summarized from Kawanishi et al. (15). Peyer's patch (PP) B lymphocytes were treated with lipopolysaccharide. When switch T cells were added, a tenfold increase was found in percentage of cells expressing IgA.

(15). Because of this apparent role, these cells have been termed *switch T cells*. Another population of T cells in GALT have been identified that can help B lymphocytes bearing surface IgA to differentiate into IgA-secreting plasma cells (16). It is not yet clear whether switch T cells and helper T cells perform their functions locally in GALT or whether they circulate and influence the B cells at the mature in the spleen or other structures. In addition to helper cells for the IgA response, a population of suppressor T cells that inhibits production of systemic immunity to orally administered antigens are present (17,18). It is thought that these cells may prevent development of potentially damaging local Arthus reactions to the many antigens present in the gut lumen. Last, a population of cells that oppose the effects of suppressor T cells has recently been described. These contrasuppressor cells inhibit suppressor T cells and also seem to encourage the development of the IgA isotype through mechanisms that are not yet understood (19). The end result of this stimulation is development of plasma cells which contain immunoglobulins that react to antigens in the gut lumen.

LYMPHOCYTE TRAFFICKING AFTER GALT STIMULATION

After antigen has stimulated IgA-precursor B lymphocytes, the B cells leave GALT and begin a long journey during which they continue the maturation process begun in GALT. First, they travel through the lymphatic drainage to mesenteric lymph nodes and then to the thoracic duct (14,20). The cells mature further in the spleen where they come under the influence of helper T cells for stimulating the IgA response and suppressor T cells to inhibit the IgG and IgM responses to the luminal antigen described above (20). Thereafter, these cells migrate through the systemic circulation to a variety of mucosal surfaces including those of the gut, bronchial mucosa, salivary glands, mammary glands, conjunctiva, and genitourinary mucosa (2,39).

The reason for this circuitous route is hypothesized to be the arming of all mucosal surfaces against an antigen present in the environment. Neonates' paltry defenses are considerably aided by the secretory IgA in the breast milk. Studies in newborn nurseries have found that babies fed breast milk have a significantly lower rate of enterotoxigenic *E. coli* diarrhea compared with their counterparts who are fed only formula; this probably reflects the presence of antibodies to maternal colonic flora in the milk (21). Similar protection has been demonstrated against cholera in breast-fed children (22). Indeed, in classic experiments performed a century ago, Ehrlich demonstrated that suckling mice from mothers immunized orally to the toxin ricin were protected against potentially lethal doses of the toxin (23). Presumably, the protection was due to secretory IgA in the milk directed to the ricin.

It is not completely settled whether antigen in the gut lumen attracts the IgA precursor B lymphocytes. Studies employing isolated ileal (Thiry-Vella) loops in rabbits have shown that mucosal immunity can be detected in loop secretions even when no antigen was directly applied to the loops (24). Therefore, the oral stimulation serves to arm a wide variety of mucosal surfaces, especially the gut, against antigens readily sampled from the environment. However, it is clear from other work that while all mucosal surfaces may be armed by an oral dose of antigen, there seems to be preferential demonstration of antigen-specific secretory IgA at the initial site of antigen administration (25).

The total time for this journey of B lymphocytes from the GALT back to the mucosa is about 4–6 days. When one stimulates an animal for the first time with oral administration of antigen, a modest response in intestinal secretions is seen in about a week to 10 days (26). However, one can prime animals with mulitple immunizing oral doses of live microorganism to give a more rapid, mucosal memory response within 4 days of rechallenge (24). Although the response is seen best with live antigen, the microorganisms need not be invasive, and similar responses have been described with immunogens such as cholera toxin which have specific binding properties (27). Thus far, it has been difficult to elicit vigorous mucosal immune responses to killed microorganisms or chemically innocuous molecules such as bovine serum albumin.

MECHANISMS BY WHICH SECRETROY IgA PROVIDES PROTECTION

Human infants usually lack immunoglobulin-containing cells in their intestinal lamina propria for the first week of life, but by the second week, IgM-containing cells predominate, although some IgA-containing plasma cells are present (Table 2) (28). After the child is 1 month of age, IgA assumes its role as the main immunoglobulin of lamina propria plasma cells. Relatively few (about 10%) IgG-containing plasma cells are normally present in the gut. The relationship between these cells and mucosal antigens is apparent by observing that during active inflammatory events, such as active ulcerative colitis, increase in all three isotypes of plasma cells is commonly seen (29).

Although the mechanisms by which secretory IgA protect the gut lumen from enteropathogens are incompletely understood, some roles for secretory IgA are clear. The major protective effect of secretory IgA results from combination of IgA with microorganisms and their toxic products in the gut lumen, thereby inhibiting attachment of these agents to the intestinal epithelium (30,31). Others have shown, however, that IgA can mediate antibody-dependent cell-mediated cytotoxicity (ADCC)–like killing with enteropathogens (*Shigella* and *Salmonella*). Last, there is some evidence

TABLE 2. *Plasma cell development in the lamina propria*

Age (mo)	IgA plasma cells	IgM plasma cells	IgG plasma cells
0–1	14	26	5
1–3	112	53	4
3–6	163	59	6
6–24	408	137	54

Data from Perkkio and Savilahti (28). Values are cells per high-power field.

that IgA can activate complement mainly via the alternative pathway (32,33). However, the significance of such activation in the gut lumen is unclear at the present time.

CELL-MEDIATED IMMUNITY IN THE GASTROINTESTINAL TRACT

In addition to humoral immunity, cell-mediated immunity is a part of the mucosal defense system. With the use of monoclonal antibodies and immunohistochemistry, it has been possible to characterize the lymphoid cells in specific anatomic compartments. By using these techniques, it became clear that interepithelial (IELs) and lamina propria lymphocytes (LPLs) are distinctive populations. The vast majority of IELs in humans are T lymphocytes, which express surface antigens associated with suppressor/cytotoxic T cells (CD8 [formerly T8 or Leu 2a]) (Table 3) (34,35). IELs have been shown to be capable of several types of cytotoxic activity including ADCC, spontaneous cell-mediated cytotoxicity (SCMC), and mitogen-induced cellular cytotoxicity (MICC) (36,37). With such capabilities, it is likely that IELs play a role in defense against viral infections of epithelial cells along the gastrointestinal tract.

The LPLs have the same 2:1 helper:suppressor (CD4:CD8) phenotype as do lymphocytes in the peripheral blood (34,35). There is good evidence from studies of human colonic mucosa that the LPLs function well in vitro with phytohemagglutinin as the mitogen (37). Overall, LPLs have similar cell populations and functional capabilities as peripheral blood lymphocytes.

Some of these cytotoxic mechanisms may function to protect the gut integrity against enteropathogens. As mentioned above, ADCC has been shown to function with secretory IgA directed against specific bacterial pathogens (*Shigella flexneri* and *Salmonella typhi*) (32,33). Although these reactions have all been demonstrated in vitro, it is tempting to speculate on the importance of a cytotoxic mechanism that involves the main secretory immunoglobulin of the gut and mucosal cells capable of cytotoxic function. A similar collaboration has been shown in experimental animals for dealing with intestinal parasites (38).

TABLE 3. *Phenotype of interepithelial lymphocytes*

	T11[a]	T4[b]	T8[b]	T4/T8	B1[a]
Small bowel	99	13.2	86.7	0.15	1
Colon	—	12.6	87.4	0.14	—

Small bowel data from Hirata et al. (35). Colon data from Selby et al. (34).

[a] Data expressed as percentage of T11 plus B1.

[b] Data expressed as percentage of T4 plus T8.

FUTURE DIRECTIONS FOR MUCOSAL IMMUNITY RESEARCH

The potentials of the mucosal immune response are only beginning to be understood. As better methods to purify populations of mucosal lymphoid cells from biopsy material become available, we will be able to begin to understand the role of the hyperplastic lymphoid tissues in many gastrointestinal inflammatory conditions. For instance, do the plasma cells in the lamina propria of inflammatory bowel disease patients play a role in the pathogenesis of that condition? Or are they merely responding to the luminal contents to which GALT is exposed following epithelial damage from some as yet unidentified agent? Does the antigen processing (M cell) mechanism of the mucosal immune system offer some clues to the pathogenesis of enteric infections and some experimental models of colonic neoplasia? Last, can we exploit the mucosal immune response for the purpose of developing oral vaccines to enteropathogens, toxins, and carcinogens that routinely pass through the gastrointestinal tract? □

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Combined Parenteral and Oral Immunization Results in an Enhanced Mucosal Immunoglobulin A Response to *Shigella flexneri*

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Achieving a vigorous secretory immunoglobulin A (IgA) response in intestinal secretions usually requires multiple doses of antigen given orally, while systemic immunity is more easily attained by parenteral immunization. This study examines the role of combined parenteral and oral immunizations to enhance the early mucosal immune response to an enteropathogen. We have used a chronically isolated intestinal-loop model in rabbits as a probe to monitor kinetically the initial (primary) local immune response to shigella lipopolysaccharide (LPS) following combinations of parenteral immunization intramuscularly (i.m.) and oral stimulation with shigellae. Predictably, effective stimulation of systemic immunity was elicited when heat-killed preparations of *Shigella* sp. strain X16 were given i.m., as shown by strong serum IgG and weak intestinal IgA activity to shigella LPS. A single oral dose of live *Shigella* sp. strain X16 given to unprimed rabbits elicited only a typical weak IgA response in intestinal secretions. However, when an i.m. dose of heat-killed shigellae was followed 1 day later by an oral dose of live *Shigella* sp. strain X16, a hyperstimulation of the early secretory IgA response was elicited, and the response reached levels found previously only after multiple oral administrations of live shigellae. This stimulation did not require the use of an adjuvant. At the same time, the animals receiving this combined oral and i.m. regimen had a lower IgG antishigella LPS activity in serum compared with their response after receiving parenteral antigen in adjuvant alone. These findings indicate that while a dichotomy exists between the systemic and mucosal immune responses, careful orchestration of the stimulatory events can promote a vigorous early local IgA response.

Since the mucosal immune system lies at the portal of entry for enteropathogens, many recent studies have concentrated on methods to prime the intestinal mucosa against these agents. Many approaches have been successful, but they usually require several weeks to achieve strong local immunoglobulin A (IgA) responses. Stimulation of the intestinal mucosal immune response to enteropathogens typically can be achieved by giving multiple oral doses of the antigen preparation or by the use of intraperitoneal priming with adjuvant. These strategies will evoke production of detectable levels of antigen-specific IgA in intestinal secretions or antigen-reactive cells in the lamina propria (3, 4, 8, 12, 30–32, 37, 40).

Recently, several groups using such immunization schedules have documented the existence of a secretory IgA mucosal memory response to enteropathogens and their toxic products in the gastrointestinal tract (1, 13–15, 19, 26). The mucosal memory responses are characterized by a more rapid and vigorous rise in the local IgA activity in response to oral challenge with antigen in primed animals than in unprimed animals. To achieve these high levels of IgA activity in secretions, multiple doses of antigen, often over a period of several weeks, are usually required. Further, in our work with *Shigella flexneri*, live antigen given orally was able to prime the animals for a mucosal memory response (13–15).

Attempts to enhance secretory IgA responses have included altering the form of the antigen, the route and schedule of administration, and the adjuvants used (15, 26). Some workers have employed combinations of parenteral injections of antigen and oral stimulation (6, 20, 29). Most often, priming of animals by parenteral immunization several days to weeks before mucosal stimulation has been ineffective in enhancing mucosal immunity (17, 40). A notable exception is the use of intraperitoneal priming, which probably causes some direct stimulation of mucosal immunity (26, 27). In the present studies, we demonstrate that giving the animals a parenteral priming with heat-killed shigellae 1 day before the oral dose of live shigellae results in an enhanced early IgA antishigella response measurable in intestinal secretions.

MATERIALS AND METHODS

Preparation of chronically isolated ileal loops in rabbits. The surgical procedure for isolating ileal loops in rabbits has been detailed previously (11). Briefly, while anesthetized, 3-kg New Zealand White rabbits have a 20-cm segment of ileum containing a grossly identifiable Peyer's patch isolated with its vascular supply intact. Silastic tubing (Dow Corning Corp., Midland, Mich.) is sewn into each end of the isolated segment, and the free ends are tunnelled subcutaneously to the nape of the neck, where they are exteriorized and secured. Intestinal continuity is restored by an end-to-end anastomosis.

About 2 ml of secretions and mucus that collect in the ileal loops is expelled daily by injecting 20 ml of air into one of the silastic tubes. The slightly opaque, colorless fluid is stored at −20°C until time of assay.

Immunization. *Shigella* sp. strain X16, a hybrid of *Escherichia coli* and *S. flexneri* which is capable of mucosal invasion but does not persist in tissues and does not cause dysentery, was used for all studies (5). Heat-killed *Shigella* sp. strain X16 was prepared by boiling overnight broth cultures for 10 min. To ensure nonviability, sample cultures were streaked onto MacConkey agar and checked for overnight growth.

Rabbits were immunized by the schedule outlined in Table

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TABLE 1. Immunization schedule

Group	n	Antigen[a]	Route	Day
1	7	Heat-killed	Peyer's patch[b]	0
2	10	Live	Oral[c]	0
3	10	Heat-killed	Intramuscular	−1
		Live	Oral[c]	0
4	11	Heat-killed, in CFA	Intramuscular[d]	−1
		Live	Oral[c]	0
5	11	Heat-killed, in CFA	Intramuscular[d]	−1

[a] All doses contained 10^{10} shigellae (*Shigella* sp. strain X16).
[b] Antigen injected into each of five Peyer's patches including the one in the isolated ileal loop.
[c] Given under mild anesthesia via nasogastric cannula.
[d] Given with CFA.

1. As in previous studies, the isolated ileal loops were created on the day prior to oral antigen administration (13–15). Although the isolated segments were not directly exposed to antigen, secretions from these loops accurately reflected the specific IgA content of the intestine due to lymphocyte recirculation (13–15). For oral immunization, the rabbits were lightly anesthetized, an orogastric tube was positioned, and the indicated dose of *Shigella* sp. strain X16 was administered. Parenteral immunization was performed by intramuscular injections at multiple sites over the hips with the heat-killed shigellae, with or without complete Freund adjuvant (CFA) as indicated.

For immunization directly into Peyer's patches, animals were anesthetized, and, under aseptic conditions, a midline abdominal incision was made; the small intestine was gently pulled out, and Peyer's patches were identified from the serosal surface. A 25-gauge needle was used to inject the heat-killed bacteria just beneath the serosal surface covering the Peyer's patches.

Enzyme-linked immunosorbent assay. A previously described enzyme-linked immunosorbent assay for detecting rabbit IgG and IgA antibodies to bacterial products was used to detect specific antibody activity in intestinal-loop secretions and serum (10). Briefly, polystyrene microdilution wells were coated with 0.1 ml of a solution containing 10 μg of *Shigella* sp. strain X16 lipopolysaccharide (LPS) Westphal preparation per ml as previously characterized (10). Immediately before the serum or intestinal secretions were tested, the LPS solution was removed and the wells were washed with phosphate-buffered saline (pH 7.2) containing 0.1% Tween 20. The sample to be assayed was diluted 1/20 in this buffer and incubated in both LPS-coated wells and uncoated wells (to control for nonspecific adsorption) for 4 h. After the wells were washed with the buffer, solutions containing either alkaline phosphatase-conjugated goat anti-rabbit IgA or alkaline phosphatase-conjugated goat anti-rabbit IgG (affinity column purified and shown to be monospecific by enzyme-linked immunosorbent assay [10]) were added to the wells and left overnight at room temperature. After an additional wash with buffer, the substrate reaction was carried out with nitrophenyl phosphate in carbonate buffer (1 mg/ml). The kinetics of the enzyme-substrate reaction were extrapolated to 100 min. The optical density at 405 nm of uncoated wells measured on a Titertek Multiscan MicroELISA Reader (Flow Laboratories, Inc., McLean, Va.) was subtracted from the optical density at 405 nm of the coated wells. Standard solutions of IgG and IgA anti-*Shigella* sp. strain X16 LPS were prepared as described previously (10) and processed daily with the unknown samples. To minimize day-to-day variation, the results of the standards were normalized and the values of the unknown specimens were corrected to these normalized standards (10). By comparing results from quantitative precipitation assays, this assay system detected 1.3 ng of specific antibody per ml and had coefficients of variation of 3.6 and 9% for IgG antishigella LPS and IgA antishigella LPS, respectively (10).

The data are presented as geometric means, since other workers have noted that such a presentation better reflects the logarithmic kinetics of the local immune response after immunization (28). The kinetics were calculated by using the $\log_{10}$ of each value for each rabbit to determine the mean, standard deviation, and standard error of the mean. For each day, the $\log_{10}$ of the standard error of the mean was added and subtracted from the mean log of specific immunoglobulin activity; antilogs of these three values were then obtained to give the geometric mean and upper and lower limits of variance about that mean. Data were statistically analyzed with the RS1 interactive data analysis system. Differences between groups on specific days were tested for significance by the Student *t* test.

RESULTS

Immunogenicity of heat-killed shigellae. Previous studies with this model system have demonstrated that oral administration of live shigellae is effective in stimulating both a primary and a memory mucosal response; however, heat-killed shigellae have been totally ineffective in stimulating a memory mucosal response (13–15). To determine whether the heat-treated preparations can be effective stimulants for a mucosal immune response, a single dose of 10^{10} heat-killed cells of *Shigella* sp. strain X16 was injected directly into each of five Peyer's patches (0.2 ml per Peyer's patch) at the time of the surgical procedure to create a chronically isolated ileal loop in the group 1 animals. The Peyer's patch in the isolated loop was one of those injected in each case. By day 4 after surgery, all seven of the rabbits so treated developed significant increases ($P < 0.01$) in the IgA antishigella LPS activity in their loop secretions over day 0 values (Fig. 1). Weak IgG activity in response to shigella LPS was detected in only a few secretions (Fig. 1).

The specific antibody activities in the serum were the opposite of those in the intestinal secretions. The IgG

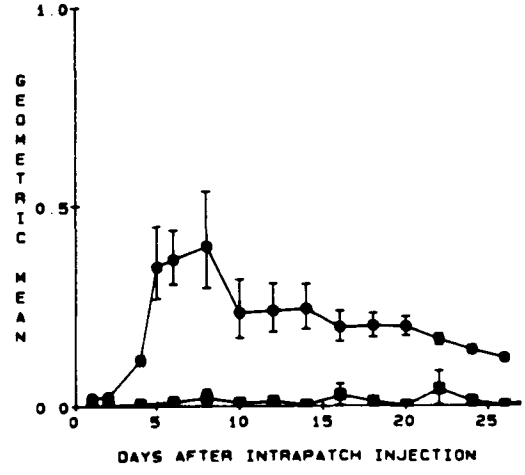

FIG. 1. Geometric mean IgA antishigella LPS (●) and IgG antishigella LPS (■) responses in isolated ileal-loop secretions from rabbits given a single injection of heat-killed shigellae into their Peyer's patches on day 0. Standard errors of the means are indicated.

TABLE 2. Serum IgG and IgA activity to shigella LPS from rabbits given antigen directly into Peyer's patches

Days postimmunization[a]	Anti-LPS activity[b]	
	IgA	IgG
6–7	0.263 (0.228–0.305)	0.479 (0.363–0.630)
8–14	0.268 (0.239–0.299)	1.038 (0.922–1.165)
15–21	0.206 (0.199–0.213)	0.927 (0.814–1.058)
22–28	0.192 (0.184–0.200)	0.824 (0.570–1.193)

[a] Data not available for preimmunization. For comparison, Tables 3 and 4 show values of 0.018 and 0.017 for IgA and 0.015 and 0.026 as geometric means of unimmunized rabbits.

[b] Data expressed as geometric means with variances as described in Materials and Methods.

antishigella LPS activity quickly increased to an overall geometric mean of 1.038, which did not significantly decline by the end of the study on day 28 (Table 2). The serum IgA activity in response to shigella LPS was weak throughout the study (Table 2). This indicates that the heat-killed shigella preparation is immunogenic for both the systemic IgG and local IgA response following immunization directly into Peyer's patches.

Immune responses following oral stimulation with live *Shigella* sp. strain X16. Group 2 rabbits received a single oral dose of 10^{10} live cells of *Shigella* sp. strain X16. This group of rabbits showed the kinetics typical of a primary local IgA response (Fig. 2) (13). The first significant increase in the IgA antishigella LPS activity over preimmunization values was found on day 6, with the response peaking on day 8. These findings are similar to those we have previously reported concerning the response following single immunization with live invasive or even noninvasive shigellae (26, 30). No IgG antishigella LPS activity was found in the intestinal secretions (Fig. 3). Further, no IgG or IgA activity in response to shigellae was detected in the serum.

Immune responses following combined parenteral immunization with heat-killed *Shigella* sp. strain X16 and oral immunization with live *Shigella* sp. strain X16. In group 3, the kinetics of the development of the local IgA response followed those of a primary mucosal immune response (Fig.

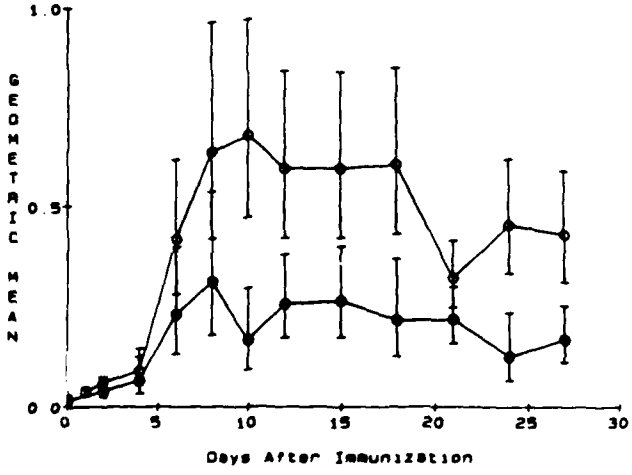

FIG. 2. Geometric mean IgA antishigella LPS responses in isolated ileal-loop secretions from rabbits given a single oral dose of live shigellae on day 0 (●) or a combined parenteral dose of antigen on day −1 and a single oral dose on day 0 (○). Standard errors of the means are indicated.

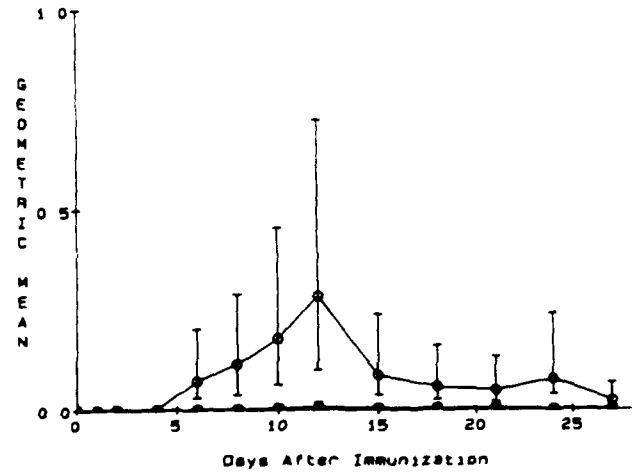

FIG. 3. Geometric mean IgG antishigella LPS responses in isolated ileal-loop secretions from the two groups of rabbits used for Fig. 2. Symbols are defined in the legend to Fig. 2. Standard errors of the means are indicated.

2). However, a significantly stronger local IgA response was found on day 10 in these rabbits compared with the response in animals receiving only a single oral dose of live shigellae (Fig. 2, group 2). The IgA antishigella activity reached by day 10 in rabbits given both parenteral and oral immunizations was also stronger than that seen in rabbits which had antigen injected directly into the Peyer's patches (group 1).

IgG antishigella activity was consistently found in intestinal secretions from these group 3 rabbits (Fig. 3). This response was more variable than the secretory IgA response, but the kinetics paralleled those of the primary secretory IgA response. By day 6 after oral immunization, the IgG antishigella in secretions had increased significantly over day 0 values, and the response peaked on day 12 (Fig. 3).

Immune responses following parenteral stimulation with heat-killed *Shigella* sp. strain X16 in CFA and oral immunization with live *Shigella* X16. Since CFA has been used to enhance and prolong both systemic and mucosal immune responses, the group 4 rabbits were used to determine whether CFA given with the heat-killed antigen would enhance or prolong the secretory IgA response to *Shigella* sp. strain X16 when the combined parenteral and oral immunization schedule was followed. As in the group 3 rabbits, a vigorous secretory IgA response was found in intestinal secretions and was significantly stronger by day 10 than that seen with the single oral dose of live shigellae (Fig. 4). There was, however, no enhancement or extension of the IgA activity compared with that of the group 3 animals. Animals which were given only parenteral heat-killed shigellae in CFA (group 5) gave a weak variable response which lagged behind the primary IgA response seen after a single oral dose of live shigellae (Fig. 4).

Weak IgG antishigella activity was found in secretions of both the group 4 and the group 5 rabbits (Fig. 5), despite the fact that these animals had high serum IgG activity in response to shigellae (Tables 3 and 4). Following a single parenteral immunization with the heat-killed shigellae in CFA, a predictable rise in the serum IgG antishigella LPS activity was seen in all animals within 1 week of immunization (Tables 3 and 4). This serum IgG activity attained maximum levels by week 3 after immunization, when it was significantly greater ($P < 0.01$) than the response by animals

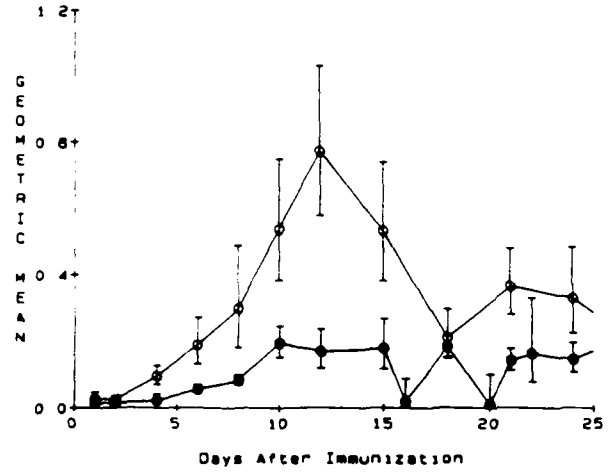

FIG. 4. Geometric mean IgA antishigella LPS responses in isolated ileal-loop secretions from rabbits given a single parenteral dose of killed shigellae in CFA (●) or a combined parenteral dose of antigen in CFA on day −1 and a single oral dose of live antigen on day 0 (○). Standard errors of the means are indicated.

given a direct injection into Peyer's patches (without adjuvant). The IgG antishigella activity in sera from animals receiving combined parenteral antigen in CFA and oral antigen (group 4) was 50% weaker than that of animals receiving parenteral antigen in CFA alone (group 5). No significant decline in this level was seen through the end of the study on day 42. Similarly, the serum IgA antishigella LPS activity quickly increased such that within 1 week all animals had a significant increase over preimmunization values (Tables 3 and 4). By two weeks, this IgA activity had attained its peak and did not decline significantly by the end of the study on day 42. Interestingly, the mean serum IgA activity in the group 5 animals was relatively low compared with that in the group 4 animals. However, these differences were not statistically significant.

DISCUSSION

The vigorous primary IgA response found in intestinal secretions in response to a combined parenteral stimulation with killed *Shigella* sp. strain X16 followed 1 day later by a single oral dose of live shigellae has not been seen previously in studies of mucosal immune responses. Earlier studies of the local IgA response to enteropathogens have shown that animals primed with three oral doses of live invasive or noninvasive shigellae will show a highly significant enhancement of their local IgA antishigella LPS response upon subsequent challenge with the same shigellae (13–15). In contrast, intragastrically administered heat-killed shigellae were totally ineffective in stimulating a mucosal memory response (15).

At least two explanations for the poor results with heat-killed shigellae are possible, and either would have important implications for vaccine preparations against enteropathogens. It is possible that particular epitopes in the killed-antigen preparations are altered in such a manner that they are no longer able to elicit a strong secretory IgA response. Alternatively, the heat-killed preparations may not be taken up effectively by the M cells which are known to exist over lymphoid follicles in the gut and which have been implicated in antigen uptake (21, 24, 34, 38). The former alternative has been examined in the present study, in which the need for M-cell processing was bypassed with the heat-killed preparations of shigellae injected directly into the Peyer's patches. Clearly, the IgA antishigella LPS activity that developed in secretions from all the rabbits in this group demonstrates that this antigen is appropriate to stimulate the mucosal immune system. Interestingly, this response occurred 2 days sooner than when the antigen was administered orally. It is most likely that the earlier inability to stimulate mucosal

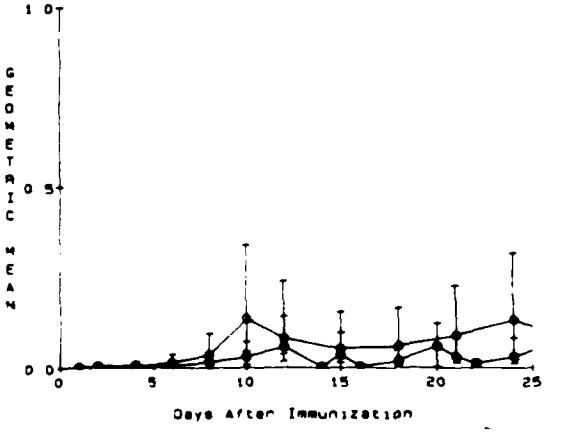

FIG. 5. Geometric mean IgG antishigella LPS responses in isolated ileal-loop secretions from the two groups of rabbits used for Fig. 4. Symbols are defined in the legend to Fig. 2. Standard errors of the means are indicated.

TABLE 3. Serum IgG and IgA antishigella LPS activity in rabbits immunized with antigen via both intramuscular with CFA and oral routes

Time[a] (days)	Activity[b]	
	IgA	IgG
Preimmunization	0.028 (0.018–0.045)	0.018 (0.010–0.033)
Postimmunization		
6–7	0.485 (0.453–0.518)	0.482 (0.445–0.522)
8–14	0.743 (0.669–0.826)	0.803 (0.762–0.846)
15–21	0.784 (0.660–0.932)	0.823 (0.817–0.933)
22–28	0.819 (0.721–0.932)	0.930 (0.862–1.003)
29–35	0.715 (0.583–0.878)	0.924 (0.831–1.027)
36–42	0.889 (0.794–0.996)	0.987 (0.930–1.029)

[a] Intramuscular immunization with heat-killed shigellae in CFA was at day −1. Oral dose of live shigellae was given on day 0.

[b] Results expressed as geometric means with the variances as described in Materials and Methods.

TABLE 4. Serum IgG and IgA activity to shigella LPS in rabbits given heat-killed shigellae in CFA intramuscularly

Time (days)	Activity[a]	
	IgA	IgG
Preimmunization	0.018 (0.011–0.028)	0.015 (0.007–0.032)
Postimmunization		
6–7	0.308 (0.180–0.525)	0.243 (0.120–0.493)
8–14	0.670 (0.494–0.908)	1.294 (1.050–1.596)
15–21	0.380 (0.494–0.908)	1.096 (0.889–1.352)
22–28	0.540 (0.450–0.647)	1.941 (1.607–2.344)
29–35	0.273 (0.166–0.448)	1.728 (1.380–2.168)
36–42	0.565 (0.469–0.681)	1.690 (1.285–2.223)

[a] Expressed as geometric means with the variances as described in Materials and Methods.

immunity with heat-killed-antigen preparations relates to their ineffective processing by the gut mucosa. Although this study provides only indirect evidence to support his contention, Owen et al. have recently reported that M cells were able to take up only viable *Vibrio cholerae* (25). Further, Wolf et al. have shown that adherence of reovirus to M cells is determined at least in part by proteins present on the surface of the virus (39). Such binding proteins may have been altered in our earlier studies by the heat treatment.

When these heat-killed preparations of shigellae in CFA were administered parenterally (groups 4 and 5), predictably strong systemic IgG and moderate systemic IgA antishigella LPS activities were found, while only weak IgG or IgA activity was detected in the loop secretions from these animals. The relatively small amount of IgG activity in intestinal secretions was not surprising. We have shown previously that IgG is not readily transported into the intestinal lumen even when there is a high titer of activity in serum (16). In the present studies, a weak but significant IgG antishigella activity was found in loop secretions from the group 3 animals. Here the combination of parenteral and oral antigen may have served to enhance the local production of IgG. The small but definite IgA antishigella LPS response in secretions of the group 5 animals demonstrates that parenteral immunization with CFA can prime the local immune response to these antigens. This finding has been demonstrated by others with a variety of antigens (6, 20, 29). This may have contributed to the total IgA antishigella activity in the groups with combined oral and parenteral immunizations.

The enhanced stimulation of the early secretory IgA response after a combined parenteral stimulation followed a day later by a single oral dose of the live bacteria could result from a number of cellular interactions. There is strong evidence that existing within gut-associated lymphoid tissues are subpopulations of regulatory T lymphocytes which can help suppress or "switch" the immunoglobulin expression by sensitized B lymphocytes after antigen has been administered orally (2, 9, 18, 22, 23, 33, 36). Further, there is good evidence that the site of antigen challenge can influence the location of the ultimate IgA response (7). In the present studies, the initial systemic antigen administration in groups 3 and 4 may have stimulated B lymphocytes, which were attracted to the antigen present in the gut after the single oral dose of antigen. The importance of the local dose is seen in group 5, in which only a weak local IgA antishigella LPS response was seen when no local antigen was given after the parenteral dose.

In addition to causing a hyperstimulation of the local IgA response, the combination of parenteral and oral doses of antigen resulted in a lower serum IgG response than when a parenteral dose of antigen was given by itself. These findings may relate to the phenomenon of oral tolerance, in which oral immunization with an antigen results in the development of suppressor T cells which inhibit the subsequent systemic response to the same antigen administered parenterally (24, 33, 35).

In the past decade, many studies have documented a dichotomy between the stimulation of the systemic and mucosal immune responses. Oral immunization will usually stimulate secretory IgA responses and can suppress subsequent systemic immunity to protein antigens and haptens. Nonetheless, the present study shows that following parenteral stimulation, the primary mucosal immune response to that antigen can be enhanced if the antigen is administered orally 1 day later. Whether this oral antigen functions to recruit circulating B immunoblasts (stimulated by the parenteral dose) to the intestine or whether it has a role in altering regulatory cells that adjust the isotypic expression and proliferation of the B cells is unclear at the present time. It is clear that this mechanism provides a useful means of rapidly sensitizing the mucosal immune system against infectious agents and their toxic products and offers a model system to better understand the basic immunology of secretory IgA.

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Autoreactivity and Altered Immune Responses in Inflammatory Bowel Disease

*David F. Keren, MD**

THE SEARCH FOR MICROORGANISMS IN INFLAMMATORY BOWEL DISEASE

The etiologic agent, or pathogenic mechanism, for the initiation of ulcerative colitis and Crohn's disease (inflammatory bowel disease, IBD) is unknown. There have been many attempts to culture candidate microorganisms from tissues of patients suffering from these diseases. Unfortunately, despite a plethora of bacteria and a few candidate viruses, no one has succeeded in consistently culturing the same creature from diseased tissues.[4] Looking for a microbial etiology is hardly a new idea. Indeed, in Crohn's original description of the disease, he suggested that a variant of *Mycobacterium tuberculosis* was the agent responsible for the disease.[13] Viral inclusions have been demonstrated in some tissue samples, and cytopathic effects can be detected in tissue culture cell lines treated with extracts of tissues from these patients.[2, 17, 36] Although some have suggested cytomegalovirus as an etiologic agent of IBD, hybridization studies have found no evidence to support this.[12, 61] Other groups have identified rheovirus-like agents, although the significance of this has been controversial.[34, 54, 71, 75]

Creative attempts to identify etiologic agents in IBD have included methods to demonstrate cell-wall defective microorganisms in involved tissues.[8, 52] However, the cell-wall defective microorganisms have not been able to reproduce IBD histopathology in experimental animals.[53]

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IMMUNOPATHOLOGIC MECHANISMS AND IBD HISTOPATHOLOGY

The failure to consistently detect a reasonable candidate microorganism as an etiologic agent resulted in investigators turning their attention to other pathogenetic mechanisms for IBD. Consequently, numerous experimental models for IBD have been suggested during the past few years. The major thesis underlying these models is that the injury observed in the mucosa is initiated by an immune-mediated mechanism.

Certainly, histopathologic features of IBD resemble those of known immunopathologic mechanisms. An Arthus reaction, for instance, is seen when pre-existing antibodies react locally with recently applied antigen. Typically, this antibody-antigen reaction occurs in the walls of blood vessels. After the antibody-antigen complexes activate complement locally, polymorphonuclear leukocytes are attracted to the area. The latter attempt to engulf the complexes, and during this process release both oxygen radicals and destructive lysosomal enzymes. These cause considerable damage to the tissue. This picture resembles that seen in classic active IBD, where acute inflammation and cryptitis are typical features (Fig. 1). Another type of immunopathologic mechanism involves cytotoxic antibodies directed to surface epithelial cells themselves. This mechanism is classically seen in Goodpasture's disease. The counterpart in the bowel may be antiepithelial cell antibodies.

IBD has a chronic inactive phase characterized by infiltration of the lamina propria by large numbers of lymphocytes and plasma cells (Fig. 2). This type of histologic picture is more characteristic of both cell-mediated and chronic humoral immune reaction. Lastly, even reaginic antibodies have been detected in patients with IBD, indicating that immediate hypersensitivity may play a role in acute episodes.

The difficulty with all of these mechanisms is that one cannot be certain whether the mechanism reveals a primary event that initiated the IBD or whether it represents subsequent secondary responses due to epithelial damage. When the gut epithelium is damaged, the myriad

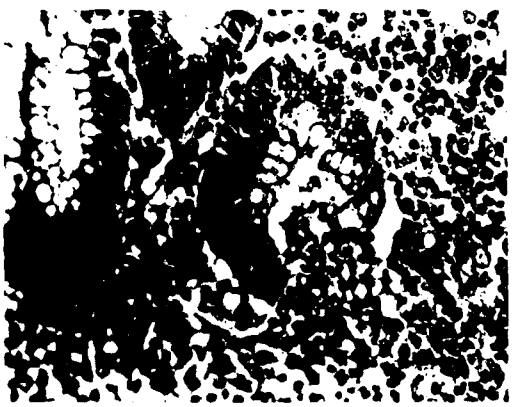

Figure 1. Active cryptitis in inflammatory bowel disease (H & E, ×500).

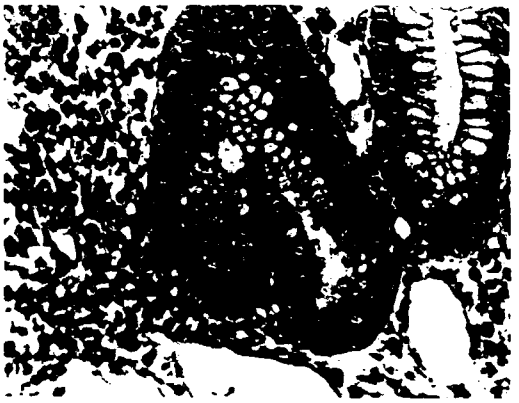

Figure 2. Chronic inactive inflammatory bowel disease. Note the forked crypt. There are considerable numbers of chronic inflammatory cells present in the lamina propria, however, acute inflammatory cells are not see (H & E, ×500).

antigens present in the gut lumen are able to enter the mucosa. This may be responsible for most of the histopathologic lesions observed.

Local Antibody Production in IBD

One consistent observation of tissues involved by chronic IBD is that the lamina propria contains markedly increased numbers of immunoglobulin-secreting plasma cells. By using immunohistochemical techniques, several groups have documented that numbers of IgG-, IgA-, and IgM-containing plasma cells are increased in the colon from patients with IBD as compared to control material.[3, 5, 37, 66, 69] The increase in immunoglobulin-containing cells correlated best with the disease activity (Table 1). However, the finding of increased numbers of plasma cells and lymphocytes serves to distinguish active chronic IBD from acute self-limited colitis.[18, 47]

Studies on defunctioned bowel indicate that the number of immunoglobulin-containing cells in the lamina propria is directly proportional to the degree of antigenic stimulation.[76] In our studies of the immunoglobulin-containing cells in IBD, we correlated the histologic measure of disease activity with the plasma cell content. Table 1 shows the comparison of these two parameters. When IBD is active, there is a significant increase in immunoglobulin-containing cells of all three major isotypes. Interestingly, when IgG-containing cells were increased, it usually resulted from focal proliferations rather than the diffuse increase seen with IgA- and IgM-containing plasma cells. Whereas IgA and IgM are both secretory immunoglobulins that respond to luminal antigens without a gross breach of the epithelium, focal IgG responses likely reflect sites of current or former cryptitis and damage to the surface epithelium. There is also a suggestion that IgE-containing plasma cells are increased in the rectal mucosa of patients with IBD. Early studies demonstrated modest increases in these IgE-containing plasma cells in the lamina propria from patients with IBD.[48] The significance of this is not clear, because immediate type hypersensitivity is not part of the clinical picture in these patients.

Table 1. *Plasma Cells in Lamina Propria of Colon Samples*[37]

HISTOPATHOLOGY	No.	IgG	IgA	IgM
Active IBD	23	45.3*	114.8	33.9
Inactive IBD	40	9.1	80.9	12.3
Reactive mucosa	39	3.9	58.7	18.9
Normal	11	2.5	68.6	2.5

* Data expressed as mean number of plasma cells per high power field.

A major problem with all studies enumerating lamina propria plasma cell content is the nonspecificity of the findings. It is highly likely that the vast majority of plasma cells present are due to nonspecific stimulation after injury to the surface epithelium of the gut rather than to some specific mucosal antigen. Although the initiating event may well involve an antibody directed to the gut mucosa, after injury to the surface epithelium, the many luminal antigens can penetrate into the lamina propria and incite a diffuse mucosal immune response. As disease activity subsides and epithelial integrity is restored, the stimulus for the local immune response gradually decreases to normal levels and the number of lamina propria plasma cells returns to a normal level. The key is to identify the initiating event for this reaction.

Antimucosal Antibodies in IBD

Several types of antimucosal antibodies have been found in these patients. Anticolon antibodies were described several years ago in patients with IBD.[7,9] These early studies found high titers of anticolon antibodies in the majority of patients with Crohn's disease while detecting similar antibodies in only about 10 per cent of controls. Unfortunately, the specificity of this antibody for IBD is far from clear. A high percentage of patients with urinary tract infections and cirrhosis also have anticolon antibodies. There does seem to be a relationship between the presence of anticolon antibodies and sclerosing cholangitis in patients with IBD. Although only about 4 per cent of patients with ulcerative colitis have primary sclerosing cholangitis,[62] these antibodies were found in 62 per cent of individuals with both sclerosing cholangitis and ulcerative colitis.[11] Although more recent studies indicate that IgG or IgA antibodies are mainly involved, earlier studies indicated that IgM antibodies are also capable of forming anticolon activity.[78] Unfortunately, although one can imagine the possible functional significance of such antibodies to damage of colonic epithelium via complement activation or via antibody-dependent cell-mediated cytotoxicity, experimental evidence has not been forthcoming to convincingly establish this point in vivo.

Other workers have found specific reactivity in the serum of most patients with Crohn's disease for rat brush border membrane antigens.[67] However, as with anticolon antibodies, these antibodies are not specific for IBD; they have also been detected in patients with *Yersinia enterocolitica* and in individuals with ulcerative proctocolitis.[68] Although antiepithelial mucosal antigen activity has been confirmed in sera from patients within IBD by a wide variety of workers,[1,26] only one laboratory

has been able to demonstrate a specific antigen. Das et al.[14] have detected a 40-kilodalton protein in colonic extracts that is specifically recognized by IgG from colon tissue of patients with IBD. This 40-kilodalton protein is present in colonic epithelium but is not present in the small intestine. Using immunoelectron microscopy, Das et al. have found this protein to be present on the surface plasma membrane. This location makes it a particularly attractive candidate as a functionally important molecule. Whereas cytoplasmic antigens or brush border antigens are unlikely to be exposed to the immune system, this 40-kilodalton antigen is. Further studies are needed to explore the ability of this antibody to mediate epithelial injury.

Other autoantibody reactivities have been seen in the sera of patients with ulcerative colitis. It is clear that these individuals have increased levels of serum IgG to casein, bovine serum albumin, alpha-lactalbumin, beta-lactoglobulin A, and 2 beta-lactoglobulin B.[39] This type of reactivity is likely due to a combination of factors. After injury to the surface epithelium, many normal food products become available as antigens to the systemic immune system. Further, as discussed later, there is a decreased suppressor T-cell response in these patients that encourages formation of antibodies. It is highly doubtful that such antibodies carry any significance in the initiation of IBD.

Experimental models for IBD have been developed that involve production of autoimmune enterocolitis by alloimmunization with mucosal antigens. By injecting a mucosal protein preparation in complete Freund's adjuvant parenterally into guinea pigs, Nemirovasky and Hugon[46] were able to produce lesions in the ileum and descending colon that resembled active IBD. With subsequent challenge either parenterally or intraintestinally, these animals developed mucosal ulcerations, congestion, and edema. Both humoral and cell-mediated immunity against the mucosal protein preparation were demonstrated. This model indicated that sensitization to mucosal antigens can result in the histopathologic picture of IBD.

Another model of IBD involved the introduction of carrageenan into the drinking water of guinea pigs for several weeks. This regimen was found to induce histologic lesions that resemble those of active IBD, including crypt abscesses, ulceration, and eventually loss of crypts.[50, 73] Although the carrageenan does not cause the disease in humans, it has proved to be a useful model for studying mucosal immunopathologic mechanisms.[43] *Bacteroides vulgatus* has been identified as the microorganism responsible for eliciting the lesions following carrageenan feeding.[49] There is no difference, however, in the ability of *Bacteroides vulgatus* isolated from patients with ulcerative colitis compared to strains from controls to elicit this response in guinea pigs.[51] The importance of this type of model is to demonstrate how a bacteria that may be encouraged by an environmental factor such as carrageenan can create mucosal damage resembling IBD.

Other experimental models employing a humoral immune mechanism to simulate IBD have explored the possible role of immune complexes in producing the characteristic lesions. The search for such models was stimulated by studies of Jewell and MacLennan,[27, 33] who

found an increased incidence of circulating immune complexes in sera of some patients with IBD. However, others have challenged that circulating immune complexes are really increased in IBD.[35] Unusual forms of complexes such as those between alkaline phosphatase and immunoglobulin and between lactic dehydrogenase and immunoglobulin have been described in patients with IBD.[42] The significance of such complexes is unclear at the present time. Formation of complexes between immunoglobulins and enzymes as well as enzyme inhibitors has been broadly described.[70, 77] Whether these complexes are related to the disease is unclear at the present time.

In rabbits, it has been demonstrated that when preformed immune complexes are injected intravenously and the rectum is irritated by a dilute formalin enema, a severe colitis results that histologically resembles acute IBD.[29, 44] The injury in these cases is likely mediated by leukocytes that are attracted to the immune complexes, which become localized in the intestinal wall. Evidence for this includes demonstration that hydroxychloroquine can prevent the experimental immune complex–mediated colitis in rabbits.[60] However, although circulating immune complexes can be found in increased frequency in patients with active colitis, the lack of demonstration of immune complexes in tissues of patients with IBD leads one to suspect that this is not a mechanism that simulates the human disorder.[22] There is evidence that polymorphonuclear leukocytes in patients with IBD are fatigued with regard to their complement-derived chemotactic factor response. When incubated in vitro with complement-derived chemotactic factors, the leukocytes from patients with IBD give a weaker response than do leukocytes from controls. This may indicate that their receptors are already bound or that they may be more primitive cells with fewer receptors available to react with these factors.[25]

Serum Immunoglobulins and Inflammatory Mediators in IBD

There is some evidence that components of complement and prostaglandins are activated in patients with IBD. For instance, in active colitis, C3 levels are elevated and there is an increased catabolism and synthesis of C3.[24, 28, 41] Both C4 and C5 activities are found to be decreased in active disease along with elevated levels of C-reactive protein.[23] In addition to alterations in the classical complement pathway, it has been found that there are increased concentrations of properdin, Clq, and properdin convertase in serum. Clq catabolism and synthesis are also increased in patients with IBD.[55]

Inhibitors of the complement system that routinely act as a counterbalance to prevent overactivation of complement by trivial stimuli are increased in patients with active IBD.[56] Increased concentrations of both C1 esterase inhibitor and of C3b inactivator have been shown in these patients. These increased levels are not surprising considering the diffuse ongoing inflammation in the gut mucosa. There is still some controversy about the complement activation in these patients. Lake et al.[41] were not able to demonstrate abnormalities in the classical pathways in these patients, and earlier studies from Ward and Eastwood[72] demonstrated normal levels of C2 in patients with active IBD. Nonetheless, the

overwhelming evidence indicates that patients with IBD have an increased synthesis and catabolism of several complement components. How much of any particular component is present at the time of measurement depends on the balance of these processes in that patient.

High serum levels of prostaglandins are a common feature in patients with ulcerative colitis.[19] It has been suggested that this increase is due to an increased rate of synthesis of prostaglandin E_2 in the colonic mucosa of these patients.[58] This increase in prostaglandin E_2 in rectal mucosa has been confirmed in organ culture systems.[20] The source of the prostaglandin E_2 is likely the mononuclear cells within these inflamed mucosal specimens.[57] It is not yet clear whether the presence of prostaglandins in rectal mucosal fluids is responsible for the disease activity, however, because Rampton and Sladen[45,59] reported that when 14 patients with active IBD were treated with prostaglandin inhibitors, while the prostaglandin E_2 rectal dialysis fluids decreased, symptoms remained.

Cellular Immune Responses in IBD

In more recent years, research has focused on cell-mediated immunity and cellular subpopulations in patients with IBD. Older studies presented evidence that systemic suppressor T-cell activity is decreased in patients with IBD.[30] As suppressor T cells are believed to be important in preventing autoimmune diseases in experimental models, the decreased numbers of suppressor T cells in patients with IBD may be related to the development of the antimucosal antibodies and lymphocytotoxic antibodies.[40] However, a more recent report on patients with mild Crohn's disease noted increased suppressor T-cell activity that correlated with lymphocytes bearing markers for monoclonal antibodies HNK-1 and Leu 21.[31] These findings may indicate that suppressor cells are being generated during the inflammatory responses in the mucosa. Alternatively, it may be that suppressor cells are normally brought into the mucosa to decrease sensitization to antigens that pass through the intestine, but in IBD the cells remain in the peripheral blood and are not brought to the mucosa. The lack of such suppressor cells along the intestinal mucosa could allow hypersensitization to a variety of mucosal antigens, which may explain the antimucosal antibodies seen in these patients. Indeed, patients with active Crohn's disease have increased numbers of spontaneous immunoglobulin-secreting cells.[65] Ironically, despite the presence of increased spontaneous immunoglobulin production, peripheral blood B-lymphocytes from patients with Crohn's disease have decreased responses to polyclonal B-cell activators such as pokeweed mitogen.

Again, a likely explanation for the variable results obtained from these studies is that suppressor activity may vary with disease activity. For instance, concanavalin A–inducible suppressor T-cell activity is decreased in patients with active IBD, but not in those with inactive disease.[38] It is again likely that the suppressor cell activities being measured relate more to the inflammation and secondary effects than they do to any initiating event for IBD.

Cytotoxic Activities in IBD

Natural killer (NK) cells are still poorly understood. They have been defined mainly on the basis of their cytotoxic effects in vitro on tumor cells, as well as on their ability to play a role in defense against viral infections.[21] In patients with IBD, it is possible that the intestinal epithelium infected by a virus may be a target of NK cells.[32] It has also been shown that NK cells may facilitate expression of Ia antigen by intestinal epithelium in experimental animals.[6, 10] Although earlier studies on the role of NK cells in intestinal epithelium have been controversial, by purifying the NK-cell population, Shanahan et al.[64] found that lamina propria NK cells had strong activity to an in vitro tumor target cell line.[64] This cell population had the surface marker for the monoclonal antibody NKH-1. A second cell population of killer cells has been identified in the mucosa. These cells are NKH-1 negative but express T11 and T8. They are cytotoxic in vitro when activated by interleukin-2 for 72 hours. Interestingly, lamina propria lymphocytes that were incubated with anti-CD3 (T3) were able to exhibit such cytotoxic activities. This activation may be unique to mucosal lymphocytes because it has not been found in studies using peripheral blood lymphocytes.[74] The role that these NK and lymphokine-activated killer cells may play in IBD is as yet unclear. However, such cytotoxic function at the delicate mucosal surface must be carefully studied. It is clear that both activities are due to heterogeneous populations that are defined by their in vitro activities.[15, 63] The fact that earlier workers did not identify NK-cell activity in mucosa from patients with IBD likely reflected the lack of a sufficiently purified population of cells.[16]

By studying the specific mucosal lymphoid responses in patients with IBD, researchers are beginning to address key issues in determining whether the autoimmune phenomenon observed in patients with IBD are primary or secondary events. The answers to these questions will likely come within the next decade. Currently available assays for antimucosal antibodies, however, do not have diagnostic or prognostic significance.

SUMMARY

Inflammatory bowel disease (IBD) is a poorly understood condition that is associated with a wide variety of immunologic alterations. Because its pathogenesis is unknown, these immunologic alterations have been investigated with an eye toward unraveling the complex mechanism of injury in the bowels of these patients. There are several lines of evidence suggesting that IBD is related to immunologic events. The histopathology of active disease resembles the Arthus reaction, whereas the presence of antiepithelial cell antibodies is reminiscent of Goodpasture's disease. Antibodies against many microorganisms and autoantibodies to mucosal components are commonly found in these patients. Further, there is a marked increase in plasma cells in the lamina propria of patients with active IBD. It is important to keep these findings in perspective. No

studies to date have been able to determine whether the features are entirely primary events, that is, related to the initial damage to the intestinal mucosa. If the surface mucosa is injured by an as-yet-unidentified agent, the immunologic findings in IBD may be secondary events. Nonetheless, the similarity in histopathology of the experimental immunologic models of IBD to the human disease encourages investigators to pursue the etiology of this complex disease.

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ENHANCEMENT OF THE SECRETORY IgA RESPONSE TO ENTEROPATHOGENS

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INTRODUCTION

In recent years, the contributions of the mucosal immune system to host defense against a wide variety of pathogenic microorganisms have been recognized. As the mucosal immune system lies at the portal of entry for enteropathogens, it has great potential strategic importance to preventing or altering the natural course of infectious diseases of the gastrointestinal, respiratory and genito-urinary tracts. Its relative inaccessibility made the mucosal immune system difficult to study in a sequential manner.

In our laboratory, we developed a model using chronically isolated loops of ileum in rabbits as a probe to follow the secretory immune response of the intestine (1). Using this model, we have characterized the intestinal IgA response to cholera toxin, Shigella flexneri, Salmonella typhi and to keyhole limpet hemocyanin (2-6). These studies have documented and confirmed several features of the local immune response relative to potential vaccine programs against enteric infections. First, an intestinal secretory IgA response to S. flexneri is best elicited by oral rather than parenteral immunization (6). Second, Peyer's patches and isolated lymphoid follices play important roles in the antigen processing in the gastrointestinal tract (2,7). Third, the major lesions seen in rabbits given pathogenic strains of S. flexneri were most frequently seen over Peyer's patches and isolated lymphoid follicles, implying that these may serve as the major site of invasion as well as initial antigen processing (3). Finally, a local secretory IgA memory response in intestinal secretions has been

elicited by oral immunization with live, locally invasive, but not with killed S. flexneri (8).

In the present studies we have concentrated on regimens to optimize the mucosal immune response to S. flexneri without using potentially pathogenic bacteria as immunogens. These studies explore the role of invasiveness and the virulence plasmid in eliciting a mucosal memory response and examine the use of adjuvants in enhancing the primary secretory IgA response of the intestine.

METHODS

Preparation of chronically isolated ileal loops

The surgical creation of ileal Thiry-Vella loops in rabbits has been described in detail previously (1). Twenty cm isolated loops containing a Peyer's patch were created in each of 8-10 rabbits per group studied. Daily secretions from the loops and weekly serum samples are stored at -20°C until time of assay.

Enzyme linked immunosorbent assay (ELISA)

The ELISA is performed as described in detail elsewhere (6). Briefly, microtiter wells are coated with a solution containing S. flexneri lipopolysaccharides (LPS) (Westphal preparation). The fluid to be assayed is diluted in phosphate-buffered saline solution (PBS) containing .05% Tween 20 (PT) and incubated in the coated and uncoated wells. These are washed and incubated with either alkaline phosphatase-conjugated sheep anti-rabbit IgA or sheep anti-rabbit IgG [both are isotype specific affinity column purified in our laboratory using methods previously described (9)]. Kinetics of the enzyme-substrate reaction are extrapolated to 100 min. Specific IgG and IgA standards are processed on each plate with the unknown fluids as previously described (9).

The data are presented as geometric means, since others have noted that this better reflects the logarithmic kinetics of the local immune response after immunization (10). These were calculated by using the $\log_{10}$ of each value for each rabbit to determine the mean, standard deviation, and standard error of the mean. For each days result, to determine the variance, the $\log_{10}$ standard error of the mean was added and subtracted

from the mean log of specific immunoglobulin activity, antilogs of these three values were then obtained to give the geometric mean with an upper and lower limit of variance about that mean. Significance was calculated using Student's t test.

Antigen and adjuvant preparations used.

Three antigen preparations were employed in these studies: 1) live S. flexneri 2457-0 (which do not usually invade, but which possess a virulence plasmid); 2) S. flexneri M4243A1 (which have been cured of the virulence plasmid and are unable to invade); and 3) Shigella X16 (which can invade surface epithelium, but is unable to persist and usually does not produce significant pathology). All of these strains were negative for producing ulceration using the Sereny test. As a positive control, the Sereny test was performed on S. flexneri strain M4243 (a virulent strain not used for immunization in the present studies).

Three adjuvant preparations were used in the present studies. The proposed mucosal adjuvants Avridine (kindly provided by Dr Keith Jensen) and DEAE-dextran were mixed with the antigen preparations as described previously (11,12). Finally, complete Freund's adjuvant (CFA) was used parenterally in combination with oral immunization in an attempt to stimulate the mucosal immune response.

RESULTS

Role of antigen form in the mucosal memory response -- invasiveness and the virulence plasmid

Our previous studies demonstrated that the locally invasive Shigella X 16 (a hybrid of S. flexneri and Escherichia coli) was able to elicit a vigorous local IgA memory response in intestinal secretions when the animal had received an oral priming with the same bacteria (8). Whereas live bacteria were able to prime the animals for this mucosal memory response, priming with heat-killed bacteria was ineffective at stimulating such a response (Fig. 1). Unfortunately, a live, locally invasive immunogen is relatively unattractive as a potential vaccine strain, even though injury resulting from the invasion is usually limited.

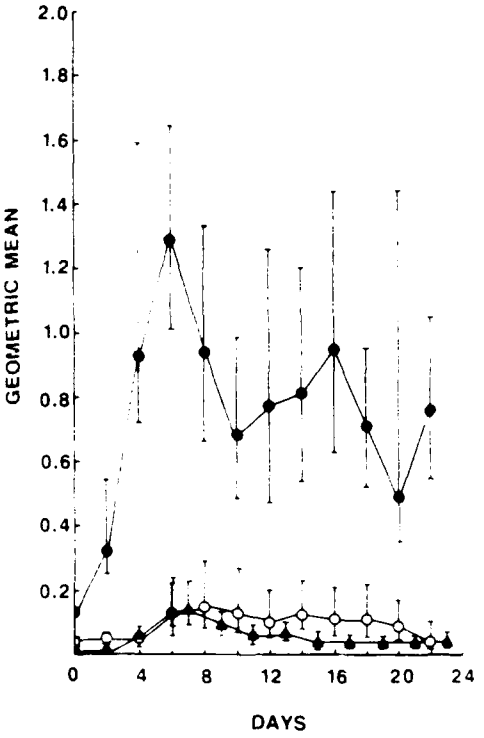

Figure 1. IgA anti-Shigella LPS activity in loop secretions from three groups of rabbits challenged with a single live oral dose of Shigella X16 on d 0. One group had not been primed (▲). A second group received three weekly oral doses of heat-killed Shigella X16 two months prior to challenge (○). The third group was primed with three weekly oral doses of live Shigella X16 (●).

Therefore, studies were performed with S. flexneri 2457-0 which is not invasive in experimental systems such as the Sereny test or rabbit intestinal loop invasion models. The primary local IgA response in intestinal secretions followed the same kinetics to those elicited by the locally invasive Shigella X16 (Fig. 2). A secretory IgA response was detectable by the sixth day after oral administration of the live strain 2457-0 (13). The overall response had a higher geometric mean peak than that of the previous work with the locally invasive Shigella X16 (13).

As with Shigella X16, when rabbits were primed with 3 weekly, oral doses of live strain 2457-0, allowed to rest for two months after the last dose, and challenged orally with the same bacteria, a striking secretory IgA memory response to S. flexneri LPS antigen was seen. By three days

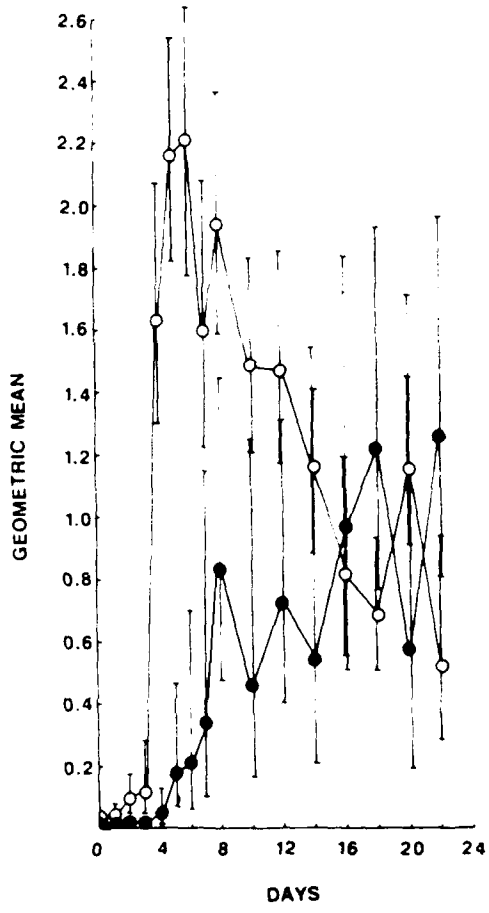

Figure 2. IgA anti-Shigella LPS activity in loop secretions from two groups of rabbits challenged with a single live oral dose of S. flexneri 2457-0 on d 0. The first group (●) had not been primed. The second group (○) was primed with three weekly oral doses of live S. flexneri 2457-0.

after oral challenge, a significantly greater local IgA response was present in the primed group than in the nonprimed group ($p < .01$) (Fig. 2) (13). The response reached its peak by day 6, at a time when the primary immune response was just beginning to be detectable.

Although S. flexneri 2457-0 is not able to invade rabbit ileum and does not give a positive Sereny test, it does contain the 140 megadalton plasmid which has been associated with virulence (14). Further, in clinical trial, some volunteers had evidence of diarrheal disease, implying that strain 2457-0 may revert to a pathogenic strain. Since this would be an undesirable event, recently, we have performed immunization studies with S. flexneri

M4243A1 which lacks the virulence plasmid (15). Our preliminary data indicate that a mucosal memory response to shigella LPS occurs with this as well (15), thus confirming that invasiveness, per se, is not a requisite for developing mucosal memory responses. As with the other studies, the local IgG responses was trivial and systemic IgG and IgA against shigella were lacking.

Role of adjuvants in enhancing the primary secretory IgA response of the intestine

Mucosal adjuvant studies. Recent publications indicated that Avridine (N,N-dioctadecyl-N',N'-[2-hydroxy-methyl] propanediamine) could increase immune responses to sheep erythrocytes, influenza B and equine encephalitis virus (11). Careful studies by Anderson demonstrate that such adjuvants as Avridine and CFA may function by increasing lymphoid traffic and inducing angiogenesis with differentiation of high endothelial venules (16). Since CFA would be unsuitable for use in the lumen of the gastrointestinal tract, we looked at the ability of our system to detect any enhancement of the mucosal immune response to shigella PS by Avridine. For these studies, a group of 10 rabbits were given a single oral dose of live shigella mixed with Avridine. Intestinal secretions from these rabbits gave similar local IgA responses to those from animals not given the Avridine (Fig. 3). No significant enhancement was found at any data point. Serum samples were uniformly negative for anti-shigella LPS activity of either the IgG or IgA class.

The findings with Avridine were similar to those with the proposed mucosal adjuvant DEAE-Dextran in our system (13). No enhancement of the secretory IgA response to a single, live oral dose of Shigella X16 was detected when the bacteria were administered orally mixed with DEAE-Dextran as described by Beh (12).

Parenteral adjuvant studies. Previous studies in our laboratory have indicated that a small primary local IgA response is detectable in secretions from the isolated ileal loops within a week after stimulation by live, oral Shigella X16. Parenteral immunizaton without adjuvant is ineffective in enhancing the local immune response to the antigens we have studied (6). Since the form of antigen is known to be of considerable importance in the development of a mucosal immune response, we have explored the role of parenterally administered heat-killed Shigella X16 in

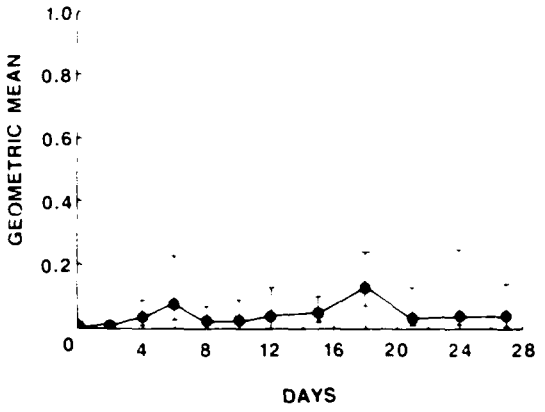

Figure 3. IgA anti-shigella LPS activity in loop secretions from rabbits given a single oral dose of live Shigella X16 which was mixed with Avridine. A typically weak primary IgA response has resulted.

complete Freund's adjuvant in stimulating the mucosal immune response. Our preliminary studies indicate that a combination of a parenteral dose of heat-killed shigella followed a day later by a live oral dose of the same bacteria results in an enhancement of the primary local IgA response.

DISCUSSION

A common goal of the many model systems used to study mucosal immunity is to optimize the stimulation of the primary and secondary IgA response. In reviewing responses of the intestine to the enteropathogen S. flexneri it was apparent that only invasive, or potentially invasive strains of shigella had elicited protection in primate studies (13). Similarly, in our earlier studies, vigorous secretory IgA memory responses to shigella LPS were readily demonstrable following oral priming with live, locally invasive Shigella X16, but not with the heat-killed preparations of the same antigen. This was true even when extraordinary high doses of the heat-killed preparations were administered orally (13). Since live shigella are able to multiply in the intestinal lumen, some felt that the heat-killed dose was inadequate. However, even when megadoses of heat-killed shigella were used, no memory response was seen.

When we used the noninvasive S. flexneri 2457-0, we found that the animals achieved a vigorous mucosal memory response, equivalent to that found in the studies with the invasive shigella (13). This latter strain

has recently been found to contain the 140 megadalton virulence plasmid which is known to be associated with invasion and disease with other strains of shigella (14). Further, some clinical studies with this strain noted occasional reversion to a pathologic state with diarrhea in some human volunteer recipients. Therefore, it was possible this 2457-0 strain which gave a negative Sereny test and a negative rabbit ileal loop invasion test may have invaded in vivo resulting in the memory response observed. To address this question, we used S. flexneri M4243A1, which lacks the 140 megadalton virulence plasmid. These studies found that this strain gave a highly significant mucosal memory response which followed the same kinetics as those of its invasive counterparts (15).

While these efforts have succeeded in enhancing the local IgA response after a long schedule of oral priming (three weekly doses with live bacteria), we were interested in methods to increase the initial primary immune response elicited. Efforts using two mucosal adjuvants in our model system failed to enhance the initial immune response to shigella LPS. However, our preliminary studies indicate that when a combination of parenteral heat-killed Shigella X16 in CFA was used followed a day later by an oral dose of live Shigella X16, a significant increase was seen in the primary IgA responses. Further studies are in progress to confirm these findings and to determine the kinetics of the development of antigen-specific lymphocytes when this combination of systemic and mucosal stimulation is used. Ability to hyperstimulate the initial mucosal IgA response would be useful information for developing vaccines which would have the ability to protect a population soon after immunization. By determining the means to manipulate the mucosal immune system, we can optimize the chances of success for clinical vaccines against enteropathogens.

Table 1. Comparison of parenteral antigen (in CFA) and oral dosage vs. oral dosage alone to elicit IgA activity in secretions to Shigella LPS

Days post-immunization	Oral and IM(CFA)[a]	Oral only[a]
0	.03	.02
4	.20	.14
8	.30	.17
12	.76	.12
16	.52	.12

[a]Activity is expressed as geometric mean (see METHODS section).

CONCLUSIONS

1. Secretory IgA memory responses can be elicited by nonpathogenic strains of shigella which lack the 140 megadalton virulence plasmid.

2. Killed forms of shigella given orally do not prime the animal for a mucosal memory response.

3. Parenteral administration of killed shigella followed one day later by a single oral dose of live shigella can significantly increase the initial response to this enteropathogen.

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Bacterial Overgrowth and Mucosal Changes in Isolated (Thiry-Vella) Ileal Loops in Rabbits

Effects of Intraluminal Antibiotics

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Atrophy of villi, with increases in crypt depth and Paneth cell number and size, occurs in chronically isolated (Thiry-Vella) ileal loops in rabbits. These loops are known to be heavily colonized with aerobic bacteria. To study the possible effect of the bacterial overgrowth, 2 experiments were performed. In the first study, two isolated ileal loops were created in each of 14 rabbits. The antibiotic loop was flushed with nonabsorbable antibiotics (neomycin, bacitracin, and gentamycin), whereas the control loop was flushed with saline. The antibiotic solution achieved a reduction in bacterial growth as compared to the loops flushed with saline. In the second study, a single ileal loop was created in each of 20 rabbits. Loops of 11 animals were flushed with an absorbable antibiotic (cefoxitin) and gentamycin, whereas those in 9 other rabbits were flushed with saline. This antibiotic combination achieved an essentially sterile loop. In both experiments, the Paneth cell population and crypt depth were less in antibiotic loops as compared to saline loops, whereas the degree of villus atrophy was nearly equal. These studies suggest a link between the overgrowth of bacteria seen in these isolated loops and the morphologic changes in the crypts.

Additional key words: Antibiotics, Villi, Crypts, Paneth cells, Bacterial flora.

Gross evidence of reactive changes in the gut after surgical procedures has received attention since Senn's animal studies of bowel resection in 1888 (29). Many of the gross, microscopic, and biochemical studies focused on possible trophic substances, either systemic or present in chyme. The possible confounding effect of bacterial infection in these studies has been recognized (16). That mucosal structure is known to be altered by normal flora, as illustrated by germ-free animal studies (1), prompted us to look at the role of bacteria in Thiry-Vella loops.

A striking hypertrophy and hyperplasia of Paneth cells, with atrophy of villi and an increase in crypt depth, occurs in chronically isolated ileal (Thiry-Vella) loops in rabbits (20). Whereas the known trophic effects of chyme, lost to the isolated loops, were suggested as an explanation for the changes seen, isolation was not the only variable characterizing the model. Within days of their creation, extensive bacterial colonization of these loops was found (21). We proposed that bacterial overgrowth might account for some of the histologic changes in isolated loops.

In the present studies, we used antibiotic solutions to control the flora of these loops and correlated the degree of bacterial suppression with changes in various morphologic parameters. Two experimental models were used. The first was of paired design with two loops per animal: an experimental loop treated with nonabsorbable antibiotics, and a saline-flushed control loop. The second study used a combination including an absorbable antibiotic, necessitating that experimental and saline control loops be maintained in different animals.

EXPERIMENTAL DESIGN

EXPERIMENT 1

In each of 15 New Zealand white rabbits were created 2 Thiry-Vella loops. Briefly, 2 20-cm segments of ileum were severed from the intestine, and the remaining ileum was anastomosed to re-establish continuity. To each end of the ileal segments (vascular supply intact), a Silastic (Dow Corning, Midland, Michigan) tube was connected; these were brought through the abdominal wall and tunnelled subcutaneously to exit at the back of the neck.

Twice a day, 9 and 15 hours apart, the loops were flushed with air to remove secretions, followed by sterile saline and air to remove mucus plugs. Into the control loop was placed 4 ml of sterile saline (285 mOsm/kg) plus 2 ml of air; to the test loop was added 4 ml of an antibiotic solution (292 mOsm/kg) plus 2 ml of air. The loop fluid obtained each morning was used to plate MacConkey agar for semiquantitative cultures. Growth on agar was graded after 24 hours incubation at 37° C; 1 = rare colony or no growth, 2 = light, 3 = moderate, 4 = heavy growth. The average weekly grades were used to characterize each animal.

The antibiotic solution (34) contained 10 mg/ml of neomycin sulfate (Prof. Vet. Lab., Belleplaine, Minnesota), and 2.5 mg/ml of bacitracin (Upjohn, Kalamazoo, Michigan) in sterile saline, stored in the dark at 4° C for not more than 3 days. Gentamycin (Schering Pharmaceuticals, Kenilworth, New Jersey) at 50 μg/ml was added for use in our last six animals, after emergency of resistant *Pseudomonas* species. These animals were histologically similar to the earlier animals. The nonabsorbable nature of these three antibiotics was a major requirement for their selection over other, more commonly used agents.

Animals were scheduled for sacrifice at 2 weeks after surgery. Tissue from both loops and a sample of normal ileum was removed, opened along the mesenteric border, and pinned for fixation in 10% neutral buffered formalin.

Experiment 2

Surgical technique, schedule and technique for flushing of loops, time of sacrifice of animals, and tissue preparation were similar to that of experiment 1, except that only 1 loop was created in each of 22 animals. Twelve animals had loops flushed with an antibiotic solution (277 mOsm/kg) containing 100 μg/ml of cefoxitin sodium (Merck, Sharp, & Dohme) and 50 μg/ml of gentamycin. Ten animals had loops flushed with sterile saline (271 mOsm/kg).

On postoperative days 2, 4, 6, 8, 10, 12, and 14, morning samples of loop fluid were processed for quantitative cultures. These included enrichment in thioglycollate broth and plating on blood agar, MacConkey agar, anaerobic laked blood agar (for detection of aerotolerant anaerobes), and Sabourad agar (for detection of fungi).

This experiment was not performed concurrently with experiment 1. Whereas animal type, age, and source were similar, some aspects of their care, including location, had changed.

Quantification of Histology

Coded histologic study utilized routine (3 to 4-μm thick) hematoxylin-eosin stained sections, and both experiments were analyzed concurrently. One section was examined for each tissue specimen.

Paneth cell hyperplasia was estimated by an ocular micrometer, with measurement of the height of the Paneth cell column (extension from crypt base into higher levels of the crypt) in 20 consecutive well-oriented crypts of each section. Crypt depth and villus height were similarly estimated by ocular micrometer, with 12 consecutive well-oriented crypt-villus pairs measured in each section. Average values were used to represent each parameter for each animal. Lamina propria heterophils (analogous to the neutrophils of humans) and epithelial mitotic figures were counted in 10 high-power fields (×400) of the crypt region. Goblet cells and intraepithelial lymphocytes (IEL) were counted per 500 epithelial nuclei of the sides of well-oriented villi.

Student's *t*-test and the paired *t*-test were used for comparisons between groups. One-sided tests were chosen.

Flora of Normal Ileum

In four unoperated New Zealand white rabbits, ileal chyme was aspirated from a site 20 cm proximal to the ileocecal valve. This was submitted for quantitative aerobic and anaerobic cultures.

RESULTS AND DISCUSSION

Data are summarized in Table 1. Statistical comparisons are listed in Table 2. For simplicity, significance tests were not adjusted for the number of comparisons made, and the possibility of apparent significance due to chance is acknowledged.

Postoperative Courses

Abscesses along the subcutaneous tract of the Silastic tubing and at the site of the peritoneal incision are common in this animal model, but in these short-term studies, did not constitute a major problem. One animal in experiment 1 died on postoperative day 13, of peritonitis secondary to an incisional hernia, and was excluded from analysis. Due to practical reasons, one animal of experiment 1 was sacrificed on postoperative day 13 and one on postoperative day 20; these animals were not otherwise different from the group sacrificed on postoperative day 14, and are included in the analysis. In experiment 2, one animal in each test condition died prior to sacrifice, apparently from respiratory infection. These were excluded from analysis. All remaining animals of experiment 2 were sacrificed on postoperative day 14.

Table 1. Mean ± Standard Error of Mean for Various Parameters[a]

Exp	Cond	Villus height (μm)	Crypt depth (μm)	Paneth cells (μm)	Mitoses (×/10 HPF)	Goblet cells (×/EN)	IEL (×/EN)	Flora week 1 (grade)	Flora week 2 (grade)
1	AL	286 ± 21	99 ± 6	55 ± 7	26 ± 4	0.120 ± 0.022	0.511 ± 0.045	1.6 ± 0.1	1.5 ± 0.1
	SL	301 ± 14	139 ± 9	79 ± 7	22 ± 3	0.137 ± 0.022	0.492 ± 0.057	3.5 ± 0.2	3.9 ± 0.1
	CI	441 ± 31	90 ± 4	24 ± 2	23 ± 5	0.051 ± 0.008	0.341 ± 0.029		
2	AL	296 ± 22	85 ± 4	38 ± 2	8 ± 1	0.117 ± 0.024	0.362 ± 0.024	See text	
	SL	288 ± 24	103 ± 9	62 ± 7	11 ± 1	0.128 ± 0.019	0.370 ± 0.022	See text	
	aCI	510 ± 35	90 ± 7	32 ± 2	17 ± 5	0.076 ± 0.007	0.322 ± 0.018		
	sCI	514 ± 36	84 ± 6	37 ± 3	13 ± 4	0.072 ± 0.011	0.297 ± 0.022		

[a] Counts of epithelial mitotic figures are expressed per 10 high power fields (HPF). Goblet cells and intraepithelial lymphocytes (IEL) are expressed per epithelial cell nucleus (EN). Grading system for bacterial flora in experiment 1 is detailed in text. AL = antibiotic loops; SL = saline loops; CI = control ileum; aCI = control ileum of antibiotic group; sCI = control ileum of saline group.

TABLE 2. STATISTICAL COMPARISONS (p VALUES)

Exp	Comparison	Villus height	Crypt depth	Paneth cells	Mitotic figures	Goblet cells	IEL	Flora week 1	Flora week 2
1	AL *vs.* CI	<0.0005	<0.05	<0.0005	NS	<0.0001	<0.001		
	SL *vs.* CI	<0.0005	<0.0005	<0.0005	NS	<0.0005	<0.05		
	AL *vs.* SL	<0.005	<0.005	<0.01	NS	NS	NS	<0.0001	<0.0001
2	AL *vs.* aCI	<0.005	NS	<0.05	NS	NS	NS		
	SL *vs.* sCI	<0.005	<0.05	<0.025	NS	<0.05	NS		
	AL *vs.* SL	NS	<0.05	<0.005	<0.05	NS	NS		
	aCI *vs.* sCI	NS	NS	NS	NS	NS	NS		

Statistical comparisons between animals and various conditions. One-sided Student's t-tests and paired T-tests were used. NS, $p > 0.05$. Other abbreviations as in Table 1.

LOOP FLORA, EXPERIMENT 1

Previous studies have shown that *Pseudomonas aeruginosa* is overwhelmingly the most prevalent bacteria in this ileal loop model. Other gram-negative bacteria were often present, but *Bacteroides* species or *Staphylococcus aureus* were rare, and anaerobes were not present (as expected in a short motile loop open to air) (21). Use of an antibiotic solution in our test loops resulted in a reduced flora relative to the control loop. As a control for methodologic technique, daily blood agar cultures were done on loop fluids from six animals; reduced flora was again found in the antibiotic loops ($p < 0.005$). The degree of bacterial control tended to be rather consistent on a daily basis within individual animals, with greater differences between animals. The saline loops consistently produced heavy growth by the end of 2 weeks.

LOOP FLORA, EXPERIMENT 2

Soon after surgical creation, the saline loops became colonized. Greater than 300,000 colonies of *P. aeruginosa* or *Escherichia coli* were typically present per ml of loop secretion. Occasionally, the anaerobic laked blood agar plate was overgrown by *E. coli*, or the Sabourad agar by *P. aeruginosa*, but anaerobes and fungi were only rarely detected, and then in very small numbers.

Antibiotic loops were essentially sterile by all culture techniques. Breakthrough growth was limited to the rare occurrence of occasional bacterial colonies on blood agar. Fungi were found in one animal on only 1 day. Anaerobes were not identified.

FLORA OF NORMAL ILEUM

Ileal chyme of four unoperated animals contained 200 to 5400 organisms per ml in aerobic culture, and 440 to 20,000 in anaerobic culture. The predominant organisms in both conditions were *Bacillus sp.* and *Corynebacterium sp.* Few coagulase-negative staphylococci and Gram-positive coccobacilli were present in one animal.

HISTOLOGIC STUDIES, EXPERIMENTS 1 AND 2

Villi of normal ileum were longer than those of either saline or antibiotic loops in both studies. The slight difference between the height of villi in saline and antibiotic loops was statistically significant only in experiment 1.

Crypt depth was increased in the saline loops compared to either the normal ileum or the antibiotic loops in both studies. The crypt depth of the antibiotic loops was indistinguishable from that of control ileum in experiment 2.

In both studies, the Paneth cell population was significantly more hyperplastic in the saline loops than in control ileum or antibiotic loops. Some Paneth cell hyperplasia was present in the antibiotic loops of both studies by comparison with control ileum, but the difference was small in experiment 2.

Differences in numbers of crypt mitotic figures were not identified in experiment 1. In experiment 2, antibiotic loops had fewer mitotic figures than saline loops. The maintenance of atrophic villi in the absence of Paneth cell hyperplasia may put little demand on epithelial kinetics.

Numbers of goblet cells per epithelial nucleus were increased in saline and antibiotic loops to a similar degree when compared with control ileum. Goblet cell hyperplasia in rabbit Thiry-Vella loops was noted in the previous study (20). Morphometrics in the present study estimated the increase to be nearly 2-fold. Antibiotic treatment and bacterial flora did not seem to affect this parameter, and the relative goblet cell hyperplasia followed the pattern of the villus atrophy. We did not notice a relationship between acute inflammation of the serosa and goblet cell hyperplasia, previously reported (20), as serosal inflammation was unusual among our animals. Furthermore, because a decrease in absorptive cell number was reflected in the measurements of villus height, an absolute increase in goblet cell numbers was slight if present.

Relative increases in intraepithelial lymphocytes present per epithelial nucleus were found in saline and antibiotic loops of experiment 1 only, although slight increases without statistical significance were seen in the loops of experiment 2. While IEL are increased in a variety of diseases (13) and seem to be a general finding with villus atrophy, they are decreased in germ-free mice (15), and in the case of co-infection by giardia and *Hexamita muris* in mice, IEL are increased without architectural changes (24). The measurement of IEL per epithelial cell in conditions of mucosal atrophy has been criticized in favor of measurement of IEL per unit of muscularis mucosa (30), but the variation in contraction/stretching in our preparations led us to favor the former method. The degree of villus atrophy must be considered in assessing changes in absolute IEL numbers of surface epithelium.

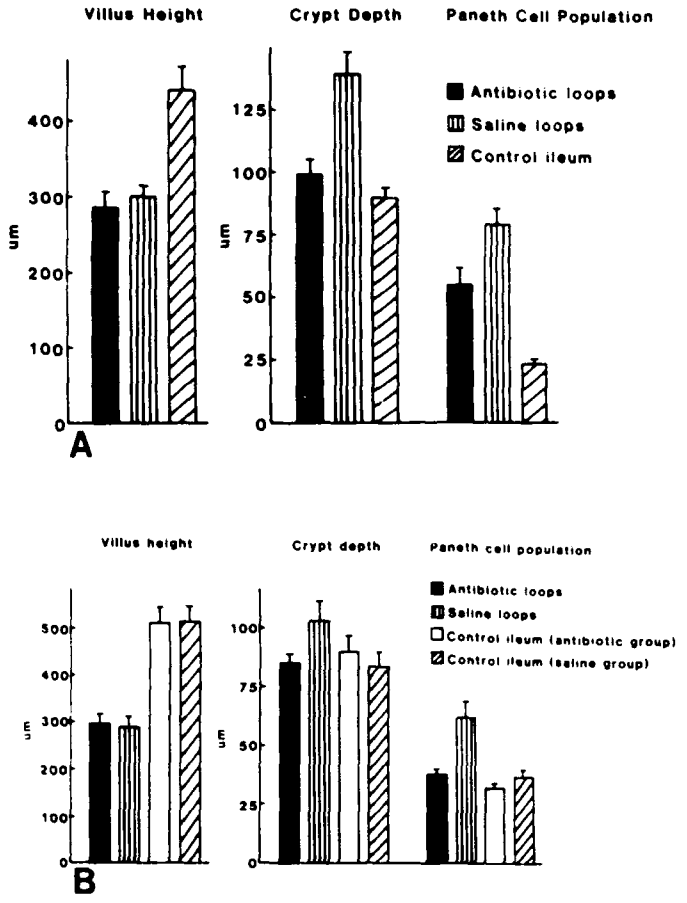

FIG. 1. Villus height, crypt depth, and Paneth cell population mean values and standard errors of means are compared. *A*, Experiment 1, nonabsorbable antibiotics. *B*, Experiment 2, including an absorbable antibiotic.

A slightly increased number of heterophils appeared to be present in the lamina propria of saline loops. Our method of quantitation was inadequate to reflect the heterophil population due to the extreme focal variability of their numbers. Broadening of the bases of villi accompanied an apparent increase in lamina propria mononuclear cells of saline and antibiotic loops.

Epithelial cell height and brush border thickness did not easily lend themselves to measurement, but both parameters appeared decreased in the Thiry-Vella loops. There was no apparent difference between saline and antibiotic loops.

Certain histologic features are illustrated in Figures 1 and 2.

DISCUSSION

The literature on small intestinal morphology and bacterial flora is composed largely of studies on blind loops and similar bowel stasis situations, and apparent inconsistencies (37) have arisen from the diversity of approaches taken. Blind or static loops can be self-emptying or self-filling, continuous with other bowel or with the external environment (Thiry-Vella) or both, chronic or acute, with heavy or light bacterial overgrowth, which may or may not be predominantly anaerobic. Such differences in characteristics affect the histology of these loops (33). There is evidence that bacteria or their secreted products (including enzymes active against brush border) contribute to the mucosal changes (22), and that antibiotic administration in many cases can effect an improvement in absorptive function (6, 22).

Isolated ileal loops in rabbits exhibit villus atrophy, crypt depth increase, and Paneth cell hyperplasia within 2 weeks of surgical creation (20). These loops contain a

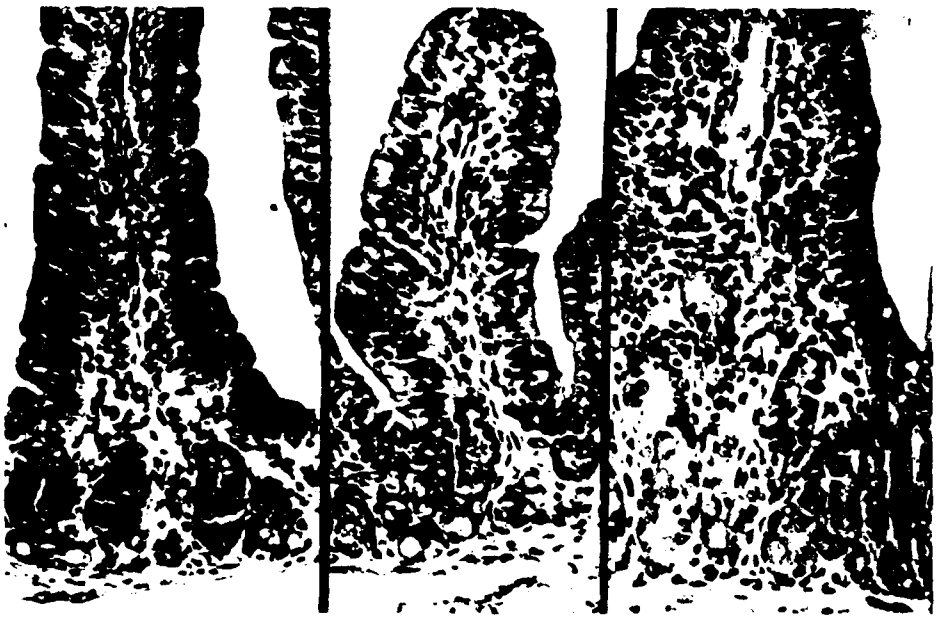

FIG. 2. Photomicrographs of control ileum (*left*), antibiotic loop (*center*), and saline loop (*right*) of one animal of experiment 1. Antibiotic treatment in this animal prevented the Paneth cell hyperplasia and increase in crypt depth seen in saline loops, but villus atrophy was unchecked. ×208.

previously characterized aerobic bacterial flora (21) and are not continuous with the intestinal chyme. We chose to study the effects of antibiotic administration on the histology of these loops.

In the present studies, villi in both antibiotic and saline loops were shorter than normal ileum, with little difference found between loops. These data agree with the work of Altmann and LeBlond (3, 4), which demonstrated that trophic factors affecting villus size were secreted in the duodenum; pancreatic secretions were found to be more important than bile. This trophic effect of chyme may explain the observation that self-emptying and self-filling blind loops can have opposite mucosal changes (7). The proximal origin of these factors in the small bowel may help maintain the gradient of villus height from duodenum to ileum, aiding the gradient inherent in the tissues (19) and effecting adaptive changes in the distal mucosa after small bowel resection (35). Both indirect (10) and direct (23) effects of nutrients on gut mass have been found, but villus atrophy was not found to be prevented by intraloop infusion of nutrient medium in the previous study of rabbit Thiry-Vella loop morphology (20). Villus atrophy in dog Thiry-Vella loops of jejunum was reportedly prevented by daily saline perfusion, but few details were provided in an abstract (18). Chyme probably has a potent trophic effect, but other factors including hormones may also effect trophic action (17). Of special interest was the absence of significant mucosal atrophy in some reports of isolated segments of small bowel, including ileal urinary conduits (32, 9).

Histologic studies of small bowel changes in blind loops and other surgical alterations have not consistently reported villus height and crypt depth separately. In a study of an experimental blind loop syndrome in rats, self-emptying loops had villus atrophy and self-filling loops had villus elongation; both conditions had an increase in crypt depth, however (33). We have presented further evidence that changes in villus height and crypt depth are not parallel processes, and that separate measurement of crypt depth provides independent information.

Antibiotic use decreased or prevented the changes in Paneth cell population and crypt depth. This effect correlated with a reduction in the bacterial colonization and was greatest with use of the more powerful combination of cefoxitin and gentamycin. Our findings recall those of Sprinz (31) two decades earlier, where he found relatively shallow crypts in germ-free animals; those monocontaminated with *E. coli* developed an increased crypt depth. While Sprinz did not comment on the Paneth cell population, in our model, morphometric analysis allowed us to conclude that the increase in crypt depth was essentially attributable to Paneth cell hyperplasia. This may help explain why this model shows an increase in crypt depth, whereas some conditions of small bowel bypass show crypt atrophy (26).

The location of Paneth cells in the crypts and the early studies showing secretion of digestive enzymes (2, 14), along with their absence in the carnivores (36), strongly associated these cells with a digestive purpose. The finding of lysozyme secretion (27) further supported this, and indeed, the chitin-cleaving property of lysozyme may explain the immense population of this cell in the Brazilian ant bear (25). Recently, intestinal phospholipase A2 and trypsin-like reactivities were identified in rat and human Paneth cells, respectively (28, 8). Separately, however, the demonstrations of phagocytosis of luminal organisms by rat Paneth cells (11, 12), and the known antibacterial actions of lysozyme (5), supported an antimicrobial role.

Hyperplasia of Paneth cells characterized the isolated loop model (20). We have reduced or prevented these changes through use of intraluminal antibiotic cocktails, suggesting that Paneth cell hyperplasia may be a response to bacterial overgrowth in a stasis situation. Whereas Paneth cell hyperplasia may often be a nonspecific response to mucosal injury, our data suggest that the bacterial flora be considered as a possible accompaniment in some conditions associated with Paneth cell hyperplasia, and that attempts to gauge possible Paneth cell hyperplasia would be useful in the evaluation of mucosal changes associated with bacterial overgrowth.

We have explored two models of intraloop antibiotic administration. The use of nonabsorbable antibiotics allows both an antibiotic loop and a saline loop to be compared within an animal, and therefore the use of the more powerful method of paired analysis. The virtual absence of flora using the combination of cefoxitin and gentamycin, however, generally was the more useful model. Development of a more effective combination of nonabsorbable antibiotics would aid these studies.

The incomplete restraint of bacterial flora with our nonabsorbable antibiotic combination provided a useful comparison in that the degree of Paneth cell hyperplasia here was also intermediate, suggesting that bacterial flora, and not a direct effect of antibiotics on the mucosa, accounted for the findings. We did not find evidence of toxic mucosal changes attributable to antibiotics by either light (present studies) or electron microscopy (as part of an ultrastructural study of ileal mucosal cells in various environments including ileal loops and tissue culture, not detailed here).

The isolated ileal loop model used here differed from the usual clinical setting of small bowel bacterial overgrowth. The environment in our model is aerobic, the range of organism type is restricted, and exogenous nutrients are not provided.

A more elaborate study, such as creation of isolated ileal loops in germ-free animals, would be necessary to directly evaluate the contribution of bacteria to the mucosal alterations in an antibiotic-free system. In our present studies, antibiotic administration prevented the Paneth cell hyperplasia which otherwise characterizes the isolated rabbit ileal loop model. We suggest that bacteria may account for some of the morphologic changes seen in the isolated loops and that the hyperplasia of Paneth cells may be a protective response to the heavy contamination by aerobic bacteria. Finally, the manipulation of Paneth cell hyperplasia by this model system offers a unique opportunity to study the function of these interesting cells.

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Models to Follow Secretory IgA Response to Mucosal Infections[1]

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Introduction

IgA was first described in 1959 and in 1965 was demonstrated to occur mainly on mucosal surfaces [1, 2]. While much experimental evidence gathered in the ensuing 2 decades has allowed us to make some general and some specific statements about this antibody and its functions in vivo, we are still in the dark about many important details of this interesting molecule. The formation of secretory IgA involves the unique collaboration between an epithelial cell which produces the 60,000-dalton glycoprotein secretory component and the plasma cell which produces the IgA. The IgA is produced by plasma cells in the lamina propria, and transported to the surface epithelium where it attaches to secretory component. It is then secreted into the lumen where the secretory component acts to protect IgA from the proteolytic environment of the gut lumen [2].

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Trafficking of Gut-Associated Lymphoid Cells

Secretory IgA response is stimulated best by antigens applied to the intestinal lumen. It is known that luminal antigen is taken up by specialized surface epithelial cells which overlie Peyer's patches, the appendix and isolated lymphoid follicles in the gut [3, 4]. This material stimulates underlying B lymphoblasts (which are enriched with precursors for developing an IgA isotype). These cells leave the gut-associated lymphoid tissues and migrate in turn to the mesenteric lymph nodes, the thoracic duct and eventually lodge in the spleen where they undergo some degree of maturation [5–8]. During their maturation, they come under the influence of switch T cells which encourage the B lymphocytes to alter their isotype from μ- to α- heavy chain [9]. Also, helper T cells which augment the IgA response are known to enhance the production of cells committed to IgA synthesis [10]. These B cells then travel to a variety of mucosal surfaces including the gastrointestinal tract, bronchial

mucosa, mammary glands and salivary glands. However, there is some evidence that the homing is influenced by the site of antigenic stimulation [11]. Further, we have learned that plasma IgA is transported into bile and then into the small intestine. Biliary tract obstruction results in decreased intestinal lumen IgA and increase in secretory IgA in plasma [12].

Mucosal Immunity to Infectious Agents: Model Systems

In the past few years, a wide variety of animal model systems have been established to study specific aspects of the mucosal immune response. Considering the diversity of the model systems, remarkably consistent information has been produced from these studies. This report reviews several model systems which have proven to be of use in studying secretory IgA responses.

Our laboratory was interested in following the kinetics of the development of secretory IgA responses against enteropathogenic bacteria and their toxic products. To allow us to collect sequential secretions, we developed a chronically isolated, ileal (Thiry-Vella) loop model in rabbits [13]. For this procedure, we operate on animals under conditions similar to human surgery. A 20-cm segment of ileum is isolated with its vascular supply intact. Silastic tubing is sewn into the two ends of the isolated loop. Intestinal continuity is restored by anastomosis. While the isolated loop remains in the peritoneal cavity, the silastic tubing is brought out through the abdominal incision and tunnelled subcutaneously to the nape of the neck where it is exteriorized and secured. The abdominal incision is closed. Daily, about 2 ml of secretions accumulate in these isolated loops. These are collected by injecting 10–20 ml of air into one end of the silastic tubing. The opaque fluid expelled from the other loop is centrifuged and the clear supernatant is stored for analysis of specific immunoglobulin content.

Using this model system we have followed the secretory IgA response to cholera toxin, *Shigella*, shiga toxin, and most recently to small carcinogens attached to carrier proteins [14–17]. Our most extensive studies took advantage of the variety of strains of *Shigella flexneri* produced in the laboratory of Dr. Samuel B. Formal at the Walter Reed Army Institute of Research.

Originally, we predicted that only strains which were invasive would be effective immunogens to be given orally for stimulating the IgA response in intestinal secretions. Therefore, for our initial studies, we looked at the sIgA response to the locally invasive hybrid of *S. flexneri* and *Escherichia coli*, *Shigella* × 16. We found that a single oral dose of live *Shigella* × 16 would elicit a weak primary IgA response which could be followed in the intestinal secretions of the isolated loop (fig. 1) [18]. Note that the loop itself never was exposed to the antigen. We were relying on the lymphocyte trafficking, described above, to provide the isolated loop with the antigen-specific cells.

Our next studies centered on proving that a secretory IgA memory response could be elicited in intestinal secretions after oral priming. This demonstration was key to development of logical oral vaccination programs. As other workers had suggested that multiple oral doses were more effective than a single dose in priming animals for mucosal immune responses, we primed rabbits by giving three weekly oral doses of *Shigella*

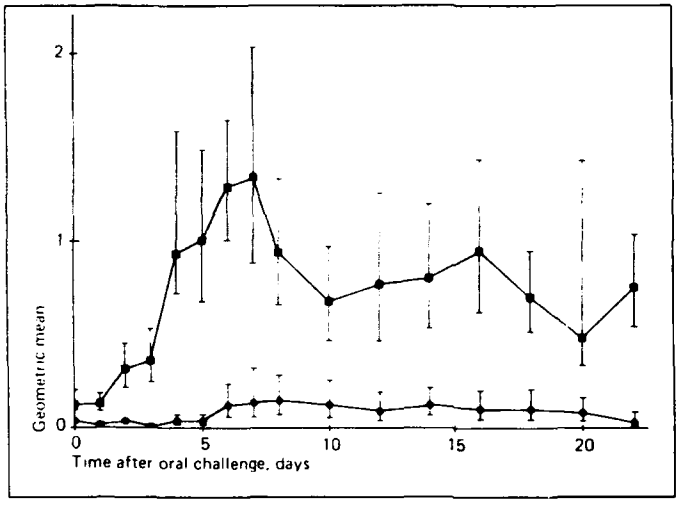

Fig. 1. IgA anti-*S. flexneri* lipapolysaccharide in ileal loop secretions following a single oral challenge with live *Shigella* × 16. The group primed with three live *Shigella* × 16 given orally 60 days prior to the challenge dose had significantly stronger responses (■) than the unprimed rabbits (●). SEM are indicated.

× 16. The animals were allowed to rest for 2 months after the third oral dose. At that time, a chronically isolated ileal loop was created and the animals were given a single oral challenge with live *Shigella* × 16. As shown in figure 1, a striking secretory IgA memory response was detected in the intestinal secretions from these animals [18]. Even 2 months after the last oral dose of antigen, there was a significant amount of IgA anti-*Shigella* lipopolysaccharide activity in the primed animals on day 0. The kinetics of the response after the oral challenge in the primed animals was remarkably strong by the 4th day.

While these studies documented the existence of a secretory IgA memory response to orally administered antigens, since the antigen used was able to invade, it would be inappropriate for use as a vaccine in humans. Therefore, we performed further studies with live, noninvasive and with heat-killed *Shigella* antigens to determine if a mucosal memory response could be elicited with safer forms of antigen given orally. Results of studies where animals were primed with three oral doses of either heat-killed *Shigella* × 16 or with noninvasive *S. flexneri* 2457-0 allowed to rest for 2 months and then challenged orally with live *Shigella* on day 0 are shown in figure 2. The heat-killed *Shigella* were totally ineffective in priming animals for a secretory IgA memory response. In contrast, the animals given the noninvasive *S. flexneri* 2457-0 strain had a marked increase in the secretory IgA activity to *S. flexneri* lipopolysaccharide [19].

Because of the poor results with heat-killed bacteria, we were concerned that the *S. flexneri* 2457-0 strain may have been able to revert to one with pathogenic capabilities since this bacteria strain contains the 140,000,000-dalton virulence plasmid responsible for invasion of the colonic epithelium. Further, in clinical trials, this strain was found to occasionally produce symptoms in volunteers receiving the vaccine orally. Therefore, we recently repeated these studies using the *S. flexneri* M4243A1 strain which lacks the 140,000,000-dalton plasmid

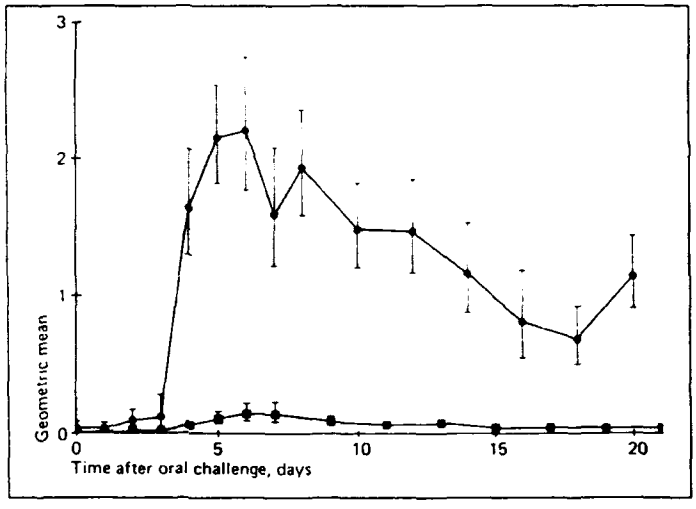

Fig. 2. IgA-anti-*S. flexneri* lipopolysaccharide in ileal loop secretions following a single oral challenge with live *S. flexneri* 2457-0. The group primed with three live oral doses of this noninvasive strain 60 days prior to the challenge dose had a significantly stronger response (●) for the first 2 weeks than animals primed with three oral doses of heat-killed *S. flexneri* (■). SEM are indicated.

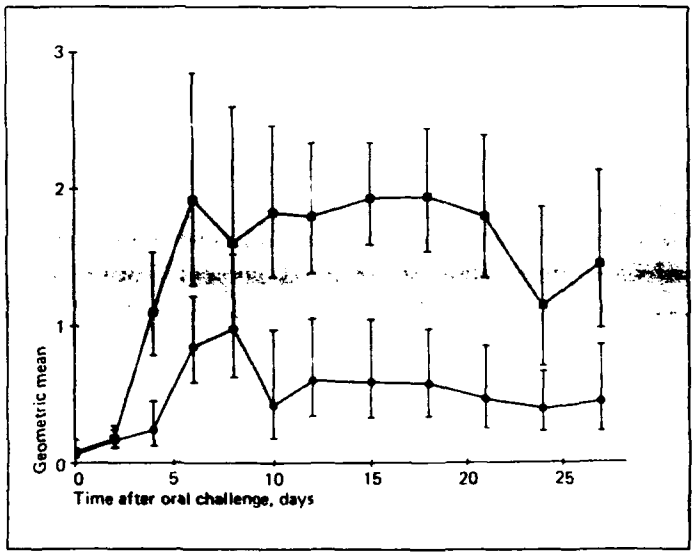

Fig. 3. IgA anti-*S. flexneri* lipopolysaccharide in ileal loop secretions following a single oral challenge with live *S. flexneri* M4243A1 which lacks the 140,000,000-dalton plasmid associated with virulence. The group primed with three live oral doses of this noninvasive strain 60 days prior to the challenge dose had significantly stronger responses (■) than did the unprimed rabbits (●). SEM are indicated.

and which thus cannot invade. As shown in figure 3, a single dose of this noninvasive strain elicited a strong primary IgA response and the triple priming, as above, elicited the strongest IgA memory response deserved to date [20]. Therefore, the secretory IgA memory response can be primed by oral immunization with live, noninvasive *Shigella*.

In our hands, in contrast, heat-killed *Shigella* were not effective immunogens to stimulate this memory response. Others, however, have succeeded in eliciting strong IgA responses with nonviable antigens. The most notable of these has been the use of cholera toxin as antigen in studies of mucosal immunity. Pierce [21] developed a model system

in which rats were given an intraperitoneal priming with cholera toxin (or various toxoids) in complete Freund's adjuvant followed by an intraduodenal challenge with the antigen. The IgA response was evaluated using an immunohistologic technique to identify cells containing specific antibody to cholera toxin in the lamina propria. They found that priming as described with cholera toxin results in a strong mucosal anticholera toxin response. Further, such mucosal immune responses were enhanced by use of a lipoidal amine adjuvant (Avridine) delivered in liposomes. We have not been able to reproduce such a positive response with our *Shigella* system [22]. Similar responses in rabbits have been reported by Pierce et al. [25] and by others. As in our studies with *Shigella,* Pierce et al. found that viable *Vibrio cholerae* elicited the most effective mucosal articholera toxin response in rabbits. It is possible that live *V. cholerae* are taken up by M cells in the mucosa and may thereby enhance presentation of secreted cholera toxin to the underlying lymphoid tissues.

A similar toxin, heat-labile *E. coli* enterotoxin, has been successfully used to raise vigorous titers of IgA antitoxin in mice. The system used by Clements et al. [24] involved oral immunization of Balb/c mice with *Salmonella typhi* containing genetic material for the synthesis of the nontoxic B subunit of the heat-labile *E. coli* [24]. Secretory IgA was collected when the animals were sacrificed and their intestinal contents removed and purified for evaluation by Elisa. The resulting mucosal IgA anti-B subunit was found to be capable of neutralizing the biologic activities of both the *E. coli* toxin and the related *V. cholera* toxin. The mucosal IgA antitoxin response was boosted by either intraperitoneal or oral doses of antibody. The disadvantage of a mouse model system is that only a single datum point can be obtained from each mouse. Although inbred mice would be expected to behave similarly to rabbits, the lack of kinetic studies limits the ability to follow the rate of mucosal antibody rise or decline in this experimental animal.

Killion and Morrison [25] used an intraperitoneal route to induce protection of C3H/HeJ mice and Saxen et al. [26] used the same route to protect rabbits from pathogenic strains of *Salmonella typhimurium.* The intraperitoneal route of immunization in experimental animals has been used successfully for many years. It is particularly effective in stimulating both an IgG and IgA response. Since the intraperitoneal route would not be used in any vaccine studies in humans, its use is limited to looking at the maximal potential of mucosal immune responses in experimental animals. For their protection studies these investigators [25, 26] injected mice intraperitoneally with dilutions of specific immunoglobulin fractions and a challenge dose of the *Salmonella.* Both IgA and IgM isotypes were found to be more protective than IgG isotypes. The form in which the antigen is presented may be particularly important in the level of protection achieved. Killion and Morrison [25] found that when lipopolysaccharide-lipid A-associated protein complexes were used to immunize mice, they were protected against challenge with a 1,000-fold lethal dose (LD_{100}) of *S. typhimurium.* Immunization with lipopolysaccharides alone, in contrast, provided significantly less protection [25].

Primates have provided important clues concerning the potential effectiveness of oral vaccines for humans. Formal et al. [27] provided critically important information with this model system. Recent work in their lab-

oratory involved the oral administration to rhesus monkeys of an invasive *E. coli* K-12 hybrid strain able to express *S. flexneri* 2a somatic antigen. As with our studies using oral immunization in rabbits with *Shigella*, the serum antibody responses were variable in the monkeys. Nonetheless, animals fed two or three weekly oral doses of live bacteria possessed significant resistance against oral challenge doses with virulent *S. flexneri* 2a 2 weeks after the last oral vaccine dose. Only 15% of 40 monkeys which received the vaccines became ill as compared to 60% of the control monkeys [27]. This work demonstrated both the potential effectiveness of the oral route to stimulate mucosal protection and showed that a non-*Shigella* carrier organism could be constructed to confer immunity to *Shigella* antigens.

Viral gastroenteritis in dogs may be caused by canine parvovirus. The virus has been responsible for massive outbreaks of severe bloody diarrhea, vomiting and death in dogs. Clinical studies on dogs with canine parvovirus gastroenteritis examined the isotype of antibodies present in feces and serum of infected dogs to determine what types of antibodies correlated with a favorable prognosis. Only intestinal antibodies to canine parvovirus correlated with resistance to gastroenteritis in this condition [28].

Bacteria and viruses have not been the only infectious agents studied for mucosal immune responses in the gastrointestinal tract. Intestinal parasites such as *Giardia lamblia* are important human pathogens, especially in immune-compromised hosts. Loftness et al. [29] used a rat model to determine whether inoculation of live *G. lamblia* trophozoites into the intestine would elicit secretory immunity to this pathogen. In their studies, anesthetized rats had a polyethylene catheter placed into the common bile duct to allow sequential sampling of biliary IgA over a 1- to 5-day period. Live trophozoites were injected directly into the proximal duodenum, or into the Peyer's patches in some rats. They found that intraduodenal injection resulted in secretory IgA in the bile against the *Giardia* by days 4–6 after the dose. Interestingly, the intrapatch injections were ineffective at stimulating specific mucosal immunity. Fluorescence studies showed that the antibody reacted with all areas of the parasite including the adhesive disk [29]. This indicates that this immunity may be able to interfere with binding of the parasite to the intestinal epithelium. The kinetics of the response to the parasite was the same as that found to *Shigella* in the rabbit Thiry-Vella loop system.

Other workers have concentrated on the fact that bile contains considerable secretory IgA. While some of the IgA clearly derives from the serum, Gilman et al. [30] found that the gallbladder is able to mount its own mucosal immune response after stimulation with microorganisms. For their work, anesthetized rabbits had *E. coli* and *Streptococcus faecalis* injected in a volume of 0.15 ml directly into the gallbladder. One week later, the bile was aspirated and tissue samples of the gallbladder were examined. Evidence of local immune response included the progressive increase in chronic inflammatory cells in the lamina propria of the gallbladder, many plasma cells among them [30]. Although the specific antibody was not determined in these studies, it was determined that the major isotype present in these cells was IgA.

The respiratory tract is another important part of the mucosal immune system which has been particularly difficult to study. Yet,

the occurrence of common influenza infections in the respiratory tract points to our need for model systems to evaluate such local immunity. To look at the mucosal immune response to influenza A virus, Liew et al. [31] used a murine system in which CBA/T6T6 mice were immunized by either aerosol or parenteral doses of Red influenza virus. Eight to eleven weeks after the immunization, they were challenged with Vic influenza virus by aerosol. They found strong protection was achieved by the mice given the aerosol priming, but not by those primed parenterally. Furthermore, protection was not correlated with specific serum antibodies or cytotoxic T cell reactivity. Rather, protection paralleled the presence of cross-reactive secretory IgA in lung washings obtained from the mice at the time of sacrifice [31]. As with studies of infectious diseases of the gastrointestinal tract, it appears that respiratory tract infectious diseases can be prevented or ameliorated by a vigorous antigen-specific local IgA response.

The mode of protection by secretory IgA seems to be prevention of attachment of the microorganism or toxic product to the mucosal surface. Recent workers, however, have begun to explore other potential modes of action of antigen-specific secretory IgA. Tagliabue et al. [32] used a combination of *Shigella*-specific secretory IgA produced in the rabbit Thiry-Vella loop model and their own antibody-dependent, cell-mediated cytotoxicity (ADCC) system with murine effector cells to look at this mode of dealing with enteropathogens [32]. They found that both macrophages and some lymphocytes (Thy 1.2-) could exert IgA-mediated ADCC when spleen was used as the source of cells, however, when gut-associated lymphoid tissue cells were used, only the lymphoid cells gave evidence of ADCC. It is unclear, as yet, whether this system operates in vivo. However, the fact that abundant secretory IgA to pathogens is present together with functional lymphoid cells in the gut mucosa causes one to seriously consider this potential mode of protective action.

Enteropathogens often elicit a polymorphonuclear inflammatory response. Therefore, the role of secretory IgA in phagocytosis has been an important point which has received some attention in recent years. Studies by Edebo et al. [33] used the mouse myeloma protein MOPC 315, a monomeric IgA directed against DNP. They attached DNP to *S. typhimurium* and found that the binding of this antibody to the bacteria enhanced the interaction with phagocytic cells. However, IgG was much more effective at removing the bacteria [34]. Importantly, studies which have used secretory IgA have found that it has had an inhibitory effect on the opsonization of bacteria. Therefore, at the mucosal surface, it is likely that secretory IgA does not collaborate with phagocytes in the uptake of bacteria. The same may not be true for parasites as Kaplan et al. [35] have recently shown that secretory IgA against *Giardia* trophozoites were able to promote parasite clearance.

Summary

The secretory IgA system lies at the portal of entry for mucosal pathogens. To study the effects of secretory IgA, many models of local immune responses to infectious diseases have been developed in the past 2 decades. They have indicated that secretory IgA is elicited to mucosal microbial pathogens and that such immunity may be protective in some circumstances. The challenge of the next decade will be to optimize the mucosal memory responses with safe vaccines and to determine the functional significance of the secretory IgA response with respect to disease prevention.

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The Enteric Immune Response to Shigella Antigens

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INTRODUCTION

The optimal regimen for stimulating a secretory IgA response in the intestine has yet to be determined. It is well known that parenteral administration of antigen will result in the formation of a systemic immune response directed to specific determinants on that antigen. Depending on the characteristics of the antigen, its dose, and the genetic capabilities of the animal, a humoral and/or cellular immune responses will result. While similar mechanisms must occur to stimulate immunity to antigens which are present in mucosal surfaces, including the gastrointestinal tract, less is known about the specific form or dose of antigens which would best elicit the production of immunity in the mucosa itself. It has been known for over a century that oral administration of antigens can elicit protection to some enteric infections. The discovery that IgA is the main antibody on mucosal surfaces provided the key for beginning definitive work to understand the biology of the mucosal immune system (Tomasi et al. 1965). While many tissues (bronchial mucosa, mammary glands, conjunctiva, genitourinary tract, biliary tract, etc.) are involved with the mucosal immune response, the gastrointestinal tract is overwhelmingly the major site of antigenic stimulation and immune response for secretory IgA (Brandtzaeg 1985). Recent studies indicate that a combination of parenteral and oral administration of antigens may enhance the initial secretory IgA response to *Shigella flexneri* (Keren et al. 1988). This review explores the mechanism for stimulation of the secretory IgA memory response to shigella lipopolysaccharide (LPS) and to Shiga toxin. Further, evidence is presented to implicate M cells in the initial mucosal ulcerations seen in dysentery.

CHRONIC INTESTINAL LOOP MODEL FOR STUDYING MUCOSAL IMMUNITY

Our Laboratory has been studying several aspects of the secretory IgA response to enteropathogens by using a chronically isolated ileal loop model in rabbits. We use this model as a probe to follow secretory IgA responses in rabbits given antigen by various routes (Keren et al. 1975). For this model, 3 kg New Zealand white rabbits (specific pathogen-free) are anesthetized with xylazine and ketamine. A midline abdominal incision is made and the terminal ileum is identified. 20 cm of ileum containing a Peyer's patch is isolated with its vascular supply intact. Silastic tubing is sewn into each end of the isolated segment. The free ends of the tubing are brought out through the midline incision and are tunneled subcutaneously to the nape of the neck where they are exteriorized and secured. Intestinal continuity is restored by an end-to-end anastomosis. The abdominal incision is closed in two layers. Each day, the secretions (2-4 ml) that collect in the ileal loop can be expelled by injecting air into one of the silastic tubings. Mucus is separated by centrifugation. The slightly opaque, colorless supernatant is available to study of specific immunoglobulin content and activity.

This chronically isolated ileal (Thiry-Vella) model system has been used to study the intestinal IgA response to cholera toxin, *Shigella flexneri*, *Salmonella typhi*, and Shiga toxin (Keren 1978; 1982; Yardley et al. 1978). This model system has established that multiple oral immunizations with live *Shigella flexneri* antigens are superior to parenteral immunization in eliciting a secretory IgA response (Keren et al. 1982b). However, by priming animals with a single parenteral dose of heat-killed shigella one day prior to oral challenge, the initial (primary) mucosal immune response can be improved (Keren et al. 1988). Since only secretions were tested in these studies, it was not completely certain that the gut-associated lymphoid tissues (GALT) were responsible for the IgA activity demonstrated. Therefore, our recent studies have examined the IgA production by GALT cells from these animals.

LOCATION OF IgA PRECURSOR B LYMPHOCYTES FOR *SHIGELLA FLEXNERI* FOLLOWING PRIMING FOR A MUCOSAL MEMORY RESPONSE

We originally predicted that only strains of shigella which were invasive would be effective immunogens when given orally for stimulating the mucosal memory response in intestinal secretions. To test this idea, we examined four strains of shigella with different invasive capabilities (Table 1). *Shigella flexneri* M4243 contains the 140 megadalton virulence plasmid, gives a positive Sereny test, and invades the intestinal epithelium. *Shigella* X16 contains the virulence plasmid and can invade the intestinal epithelium, however, it does not replicate following this invasion and does not give a positive Sereny test. *S. flexneri* 2457-0 contains the virulence plasmid, but does not invade the surface epithelium and does not give a positive Sereny test. The last strain studied, *S. flexneri* M4243A$_1$ lacks the virulence plasmid and does not invade the intestinal epithelium. Surprisingly, when administered orally, all four strains were able to elicit vigorous mucosal memory responses (Keren et al. 1985; 1986). Indeed, the strongest response was elicited by the avirulent M4243A$_1$ strain (Keren et al. 1986).

Table I. Characteristics of Shigella used in the present studies

Strain	Virulence plasmid	Sereny test	Intestinal invasion
S. flexneri M4243	+	+	+
Shigella X16	+	o	+
S. flexneri 2457-0	+	o	o
S. flexneri M4243A$_1$	o	o	o

Since all four strains could elicit a mucosal memory response, we wished to determine the location of specific antigen-reactive cells following different priming regimens and to establish their migration pattern after oral challenge. In these studies, we used *S. flexneri* M4243A$_1$ for immunization. Three intragastric immunizations were given one week apart at 74, 67, and 60 days prior to dissection. One to ten days before dissection, the rabbits were given a single oral dose of live *S. flexneri* M4243A$_1$. The rabbits were sacrificed and the lymphoid populations in the spleen, Peyer's patches, and

mesenteric lymph nodes were sampled. Tissues were cut into 1 cm^3 fragments with a sterile blade and placed in cold RPMI-1640. The cells were carefully teased apart and passed through the steel mesh. This material was centrifuged at 400 x *g* at room temperature for 7 minutes. The pellet was gently resuspended and washed twice in RPMI 1640 medium. The total number of cells and their viability were determined. 4×10^6 mononuclear cells in 1 ml tissue culture medium were added to each well of 24 well polystyrene tissue culture plates with flat bottom wells (Costar). Cultures were placed in a humidified, 5% CO_2, 37°C incubator. At the times indicated (days in culture), three wells for each tissue were aspirated. Cellular debris was removed by centrifugation at 400 x *g* for 5 minutes and the supernatants were stored at -20°C until they were assayed. Assays were performed using a previously described ELISA specific for IgA and IgG antibodies to *S. flexneri* LPS (Keren 1979).

In Tables 2,3, and 4 are shown the IgA anti-Shigella LPS responses from rabbit Peyer's patches, mesenteric lymph node, and spleen, respectively. It is clear from these data that as early as one day following rechallenge with live M4284A1, IgA-specific B lymphocytes are present within Peyer's patches. By the fourth day following rechallenge, a significant increase is seen in the amount of antigen-specific IgA produced. Strikingly, by the fifth day, B cells have left the Peyer's patches only to return by the tenth day following rechallenge. Indeed, by day ten, considerable antigen-specific IgA can be detected as soon as the second day in tissue culture. This implies that the cells present in Peyer's patches were present in considerably greater numbers at the later times.

In contrast, the lymphocytes from mesenteric lymph nodes from the first day following rechallenge were unable to produce specific IgA anti-Shigella LPS. By the third postchallenge day, there was a dramatic increase in the IgA anti-Shigella LPS which persisted through the fourth day in mesenteric lymph node cells. However, by the fifth day postchallenge these responses had returned to baseline values indicating that the cells traveled from the Peyer's patches to the mesenteric lymph nodes within three days following oral rechallenge. They left this station such that by day five postchallenge little specific activity to Shigella LPS was detectable. It is likely that these cells then traveled to the spleen as cultures from splenic mononuclear cells on days one and three show virtually no IgA anti-Shigella LPS activity, while the cultures from day four show significant IgA anti-Shigella LPS activity (Table 4). The period of time within the spleen is very brief as the activity declined by day five to almost baseline values. Beyond day six, no significant IgA anti-Shigella LPS activity was detectable.

When these same tissues were examined in an unprimed rabbit, no significant IgA anti-Shigella LPS activity was detectable from any tissue on any day. Studies of the IgG content of these supernatants have been most instructive. Consistent with our former *in vivo* data indicating that little IgG anti-Shigella LPS is present in intestinal secretions (Keren et al. 1978; Keren et al. 1986), our present studies found little specific IgG produced in the culture supernatants from the mononuclear cell preparations. This indicates that the responses seen in the secretions do not merely reflect preferential uptake of systemic polymeric IgA by intestinal epithelial cells with subsequent transport into the gut lumen. Rather, they accurately reflect the capabilities of the mononuclear cells stimulated following oral antigen administration to produce an IgA as opposed to an IgG response to *Shigella flexneri* antigens.

Table 2. IgA anti-Shigella LPS in Peyer's patch supernatant

Days in culture	Day after challenge					
	1	3	4	5	6	10
0	0.008	0.090	0.000	0.003	0.014	0.002
1	0.016	0.035	0.078	0.017	0.047	0.003
2	0.034	0.012	0.083	0.009	0.061	0.963
3	0.120	0.019	0.173	0.004	0.001	1.963
4	0.119	0.024	0.367	0.063	0.351	1.255
5	0.077	0.059	0.479	0.128	0.440	2.112
6	0.186	0.146	0.503	0.085		2.005
7	0.245	0.000	0.667	0.009	0.093	1.797
14	0.241	0.026	1.284	0.055	0.424	1.371
21	0.438	0.332	1.871	0.063	0.815	1.107

Table 3. IgA anti-Shigella LPS in ~~spleen~~ mesenteric lymph node supernatant

Days in culture	Day after challenge					
	1	3	4	5	6	10
0	0.000	0.051	0.000	0.008	0.012	0.004
1	0.006	0.544	0.644	0.008	0.049	0.002
2	0.009	0.500	0.326	0.001	0.036	0.009
3	0.002	1.770	0.375	0.027		0.008
4	0.006	0.664	0.433	0.008	0.097	0.007
5	0.022	1.693	0.544	0.027	0.000	0.006
6	0.008	1.061	0.464	0.002		0.009
7		0.434	0.377	0.001	0.042	0.010
14	0.030	0.910	0.727	0.026	0.082	0.021
21	0.016	2.273	0.413	0.021	0.033	0.005

Table 4. IgA anti-Shigella LPS in spleen supernatant

Days in culture	Day after challenge					
	1	3	4	5	6	10
0	0.000	0.053	0.000	0.008	0.000	0.002
1	0.000	0.022	0.271	0.013	0.001	0.000
2	0.006	0.015	0.364	0.000	0.000	0.003
3	0.000	0.025	0.372	0.021	0.006	0.010
4		0.032	0.673	0.013	0.039	0.014
5	0.001	0.088	0.740	0.048	0.010	0.026
6	0.014	0.107		0.176		0.012
7	0.010			0.116	0.007	0.038
14	0.040	0.050	1.134	0.076		0.019
21	0.034	0.056	0.878	0.058	0.007	0.034

* Results expressed as ΔOD_{405nm} / 100 min

M CELL UPTAKE OF SHIGELLA AND THE MUCOSAL IMMUNE RESPONSE

Since our previous studies demonstrated that oral or intraloop immunizations of all four strains of *S. flexneri* (Table 1) were able to elicit a vigorous primary mucosal immune response and a mucosal memory response, we hypothesized that these strains must be processed in a similar manner by the GALT. We know from the work of Owen (1977) that there are specialized surface epithelial cells which sample intraluminal antigens including microorganisms (Bockman and Cooper 1973; Rosner and Keren 1984). It is clear that M cells respond to microorganisms present within the gut lumen. A recent study by Smith et al. (1987) demonstrated that a significant maturation and increase in the number of M cells occurs when specific pathogen-free mice were transferred to a normal animal house environment.

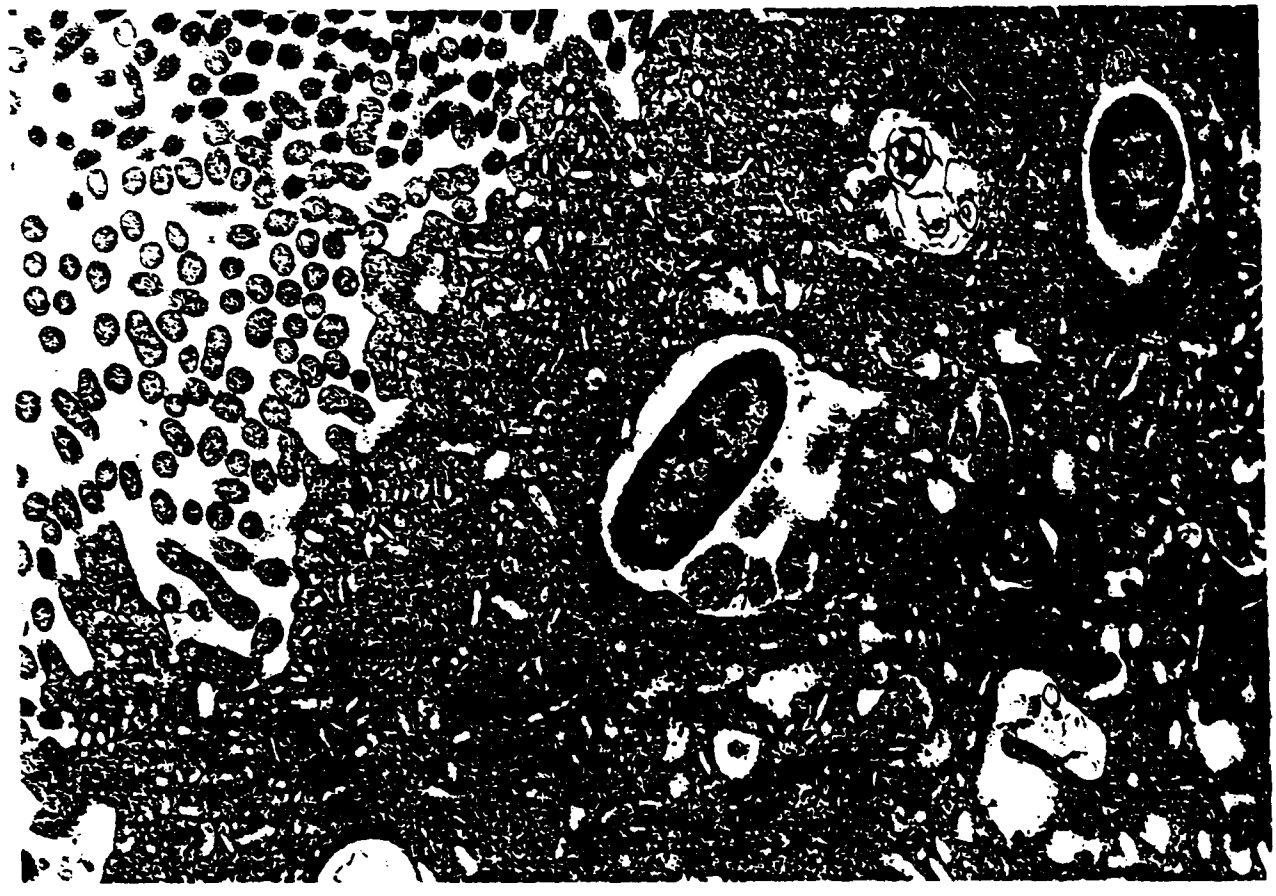

Fig. 1. Electron photomicrograph showing two Shigellae within an M cell

To determine how *S. flexneri* are processed by the intestinal epithelium, we allowed the four strains of Shigella listed in Table 1 to incubate for 90 minutes or 18 hours in acutely ligated loops of rabbit ileum. All four strains of Shigella showed readily demonstrable uptake over the dome regions of the Peyer's patches. This was assessed in two ways. First, ultrastructural studies were performed to demonstrate the bacteria within the M cells in the follicle-associated epithelium (Fig. 1). Then, frozen sections of rabbit ileum were obtained through Peyer's patches and adjacent villus epithelium. These sections were stained with Giemsa stain to demonstrate the Shigella (Fig. 2). The frozen sections allowed us to perform an accurate count of the bacteria. These counts were performed

with the aid of the Bioquant Biometrics Image Analyzer (Nashville, Tennessee) with an IBM computer used to measure the actual length in millimeters of the lining epithelium over the villi and over the dome regions of the Peyer's patches. The average of 100 areas for dome and villus areas from representative rabbits was calculated. This allowed us to directly express data as bacteria/mm^2 of surface epithelium. Further, it permitted a direct comparison of villus surface area to follicle-associated epithelium surface area. To be included in a count, we required that the entire Shigella be located within the cytoplasm. By focusing up and down, a vacuole was usually discernable around each engulfed bacterium (Fig. 2). Bacteria which were adherent to the surface epithelium but which were not clearly present within the cytoplasm were not counted.

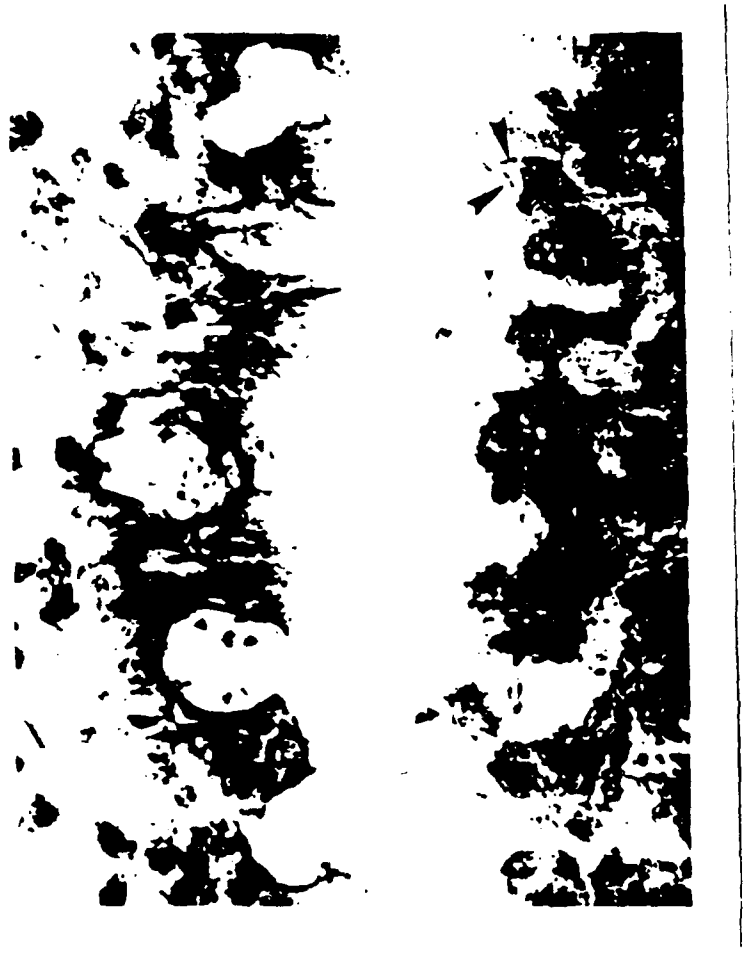

Fig. 2. Photomicrograph depicting Shigellae (arrows) within the surface epithelium

The bacteria seen at the 90 minute time period by ultrastructural studies were contained within membrane lined vesicles (Fig.1), although some vesicles in the loops given the pathogenic strain (*S. flexneri* M4243) showed early evidence of breakdown of the vesicles. The three nonpathogenic strains (Sereny test-negative) were taken up with equal efficiency regardless of their invasive capabilities or of the presence of the 140 megadalton virulence plasmid. All strains examined had relatively few Shigella (ten fold less) within the villus epithelium as compared to the follicle-associated epithelium. The pathogenic strain *S. flexneri* M4243 had significantly greater uptake of the bacteria in the dome regions than did the three nonpathogenic strains (Fig. 3).

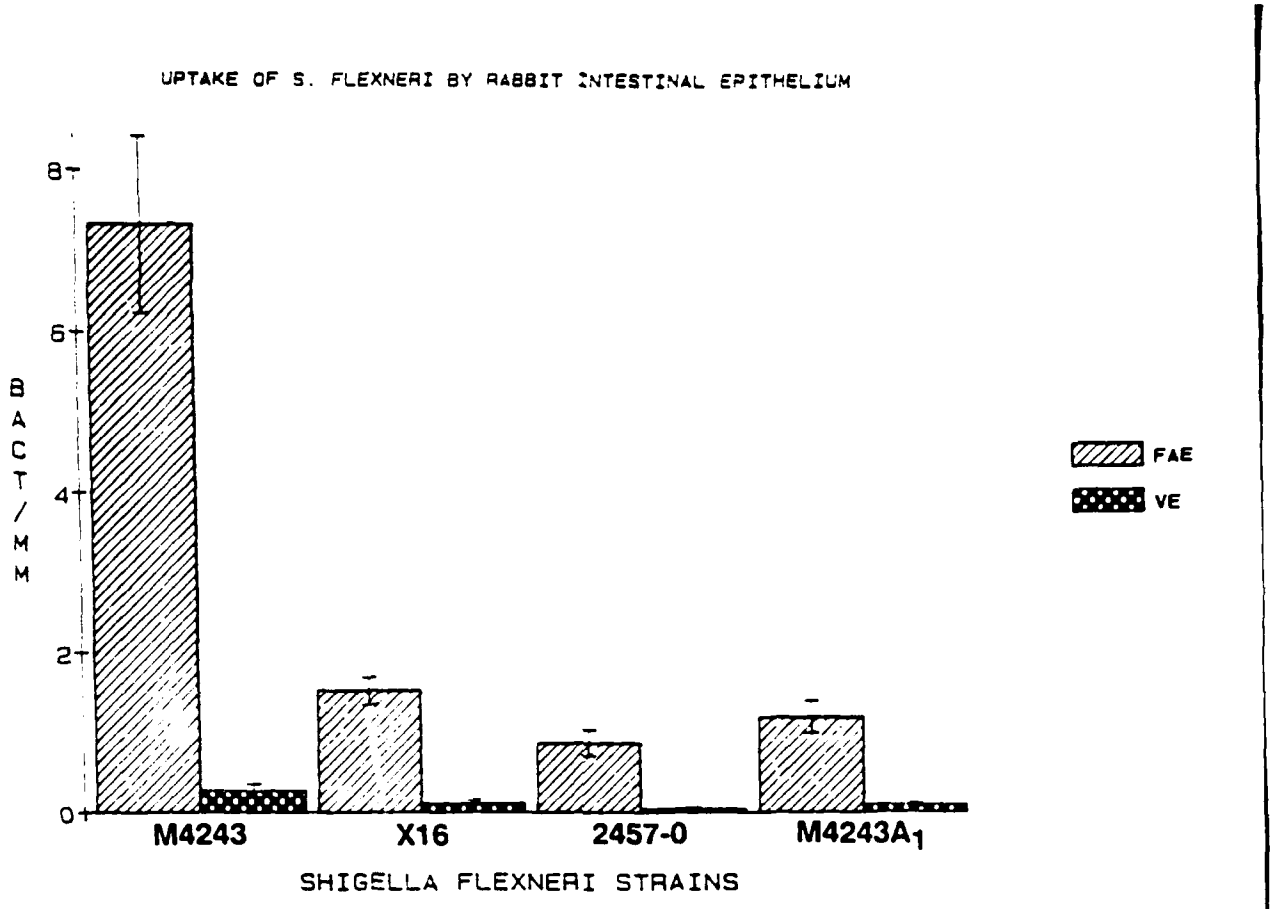

Fig. 3. Uptake of Shigellae by follicle-associated epithelium (FAE) and villus epithelium (VE)

Both pathogenic and nonpathogenic strains of Shigella were taken up preferentially by the specialized M cells in the follicle-associated epithelium as opposed to the villus epithelium; therefore, M cells do not distinguish between Shigella on the basis of expression of antigens encoded by the virulence plasmid. Since all four strains have been found to elicit significant mucosal immune responses in our previous studies where direct intraintestinal stimulation was given to chronically isolated ileal loops and since the three non-invasive strains could prime rabbits for mucosal memory response regardless of their ability to invade surface epithelium or the presence of the 140 megadalton virulence plasmid, we believed that the strains would be sampled with equal efficiency by the surface M cells. The findings in these acute loop studies are consistent with this hypothesis. There was, however, a significant difference of the uptake of the pathogenic *S. flexneri* M4243 strain versus the avirulent strains at 90 minutes. This likely reflected the successful replication by the latter bacteria within the tissue following uptake. Therefore, we followed this process for 18 hours to allow replication to continue and pathologic events to occur. After 18 hours of incubation, profound mucosal ulceration was seen exclusively with the *S. flexneri* M4243 strain. The acute loops incubated for 18 hours with these bacteria showed a hemorrhagic surface with marked acute inflammation throughout the lamina propria. Ulceration was present predominantly in the dome regions over the Peyer's patches. Although there was mucosal damage in the adjacent villi, the surface epithelium was, in general, intact. With the pathogenic M4243 strain, myriads of microorganisms were seen in the exudate over the ulcer and within the tissues, attesting to their successful replication.

In marked contrast, Shigellae were not found within the surface epithelium of acute loops incubated with the noninvasive strains for this time. Further, the three nonpathogenic strains showed no ulceration after the 18 hour incubation. With the *Shigella* X16 strain, there was some hemorrhage in the lumen. However, the epithelium overlying the villi and the dome regions of Peyer's patches in animals given the *Shigella* X16 strain was intact.

These findings indicate that in addition to being the site for antigen sampling , M cells serve as the portal of entry for pathogenic microorganisms. Indeed, M cells have been proposed by others as a portal of entry for intestinal pathogens including the human immunodeficiency virus (Sneller and Strober 1986).

MUCOSAL IMMUNE RESPONSE TO SHIGA TOXIN: FUNCTIONAL SIGNIFICANCE

While invasion with replication in the mucosa is the main mechanism in the pathogenesis of dysentery, it has been known for some time that *Shigella dysenteriae* 1 produces a protein cytotoxin, Shiga toxin, which in tissue culture inhibits protein synthesis in specific cell lines (Sandvig 1987; Brown 1980; Eiklid and Olsnes 1980). Since Shiga toxin must first bind to a glycolipid receptor before it can initiate cell change *in vitro* (Brown et al. 1983), the presence of an antibody to interfere with this binding would theoretically interfere with the cytotoxicity process.

Vigorous mucosal immune responses can be elicited to other enterotoxins (Yardley et al. 1978; Pierce et al. 1983). Studies using the present Thiry-Vella loop model system have stressed that cholera toxin is a most potent mucosal immunogen (Yardley et al., 1978). In the present studies, we immunized two rabbits intraloop with a preparation of Shiga toxin provided by Dr. J. Edward Brown. The results in Fig. 4 show the specific intestinal antibody activity from two rabbits given direct intraloop immunization with Shiga toxin. The Shiga toxin was given on days 0, 7, and 14. We found that by day 10, a significant increase in the IgA anti-Shiga toxin activity over background had occurred. Booster doses of Shiga toxin given on days 7 and 14 had the effect of decreasing both the IgA anti-Shiga toxin activity in the following day's secretion as well as decreasing the inhibition titer of the loop secretions performed in the HeLa cell assay. This was due to binding of specific IgA in these secretions by the Shiga toxin. Both doses were followed by substantial increased IgA activity in subsequent days. There was an excellent correlation of the IgA anti-Shiga toxin activity with the inhibition titer. Little IgG anti-Shiga toxin was detected in these intestinal secretions. Future studies will attempt to confirm these results in a larger group of animals and to establish whether a secretory IgA memory response against Shiga toxin can be elicited. It is notable, however, that Shiga toxin is second only to cholera toxin in being able to elicit a vigorous secretory IgA response to our model system. Since it is clear that IgA responses are highly dependent on T cell control mechanisms (Kawanishi et al. 1983; Campbell and Vose 1985), it may be worthwhile to study the mechanism of Shiga toxin mucosal stimulation to determine how it interacts with helper or switch T cells.

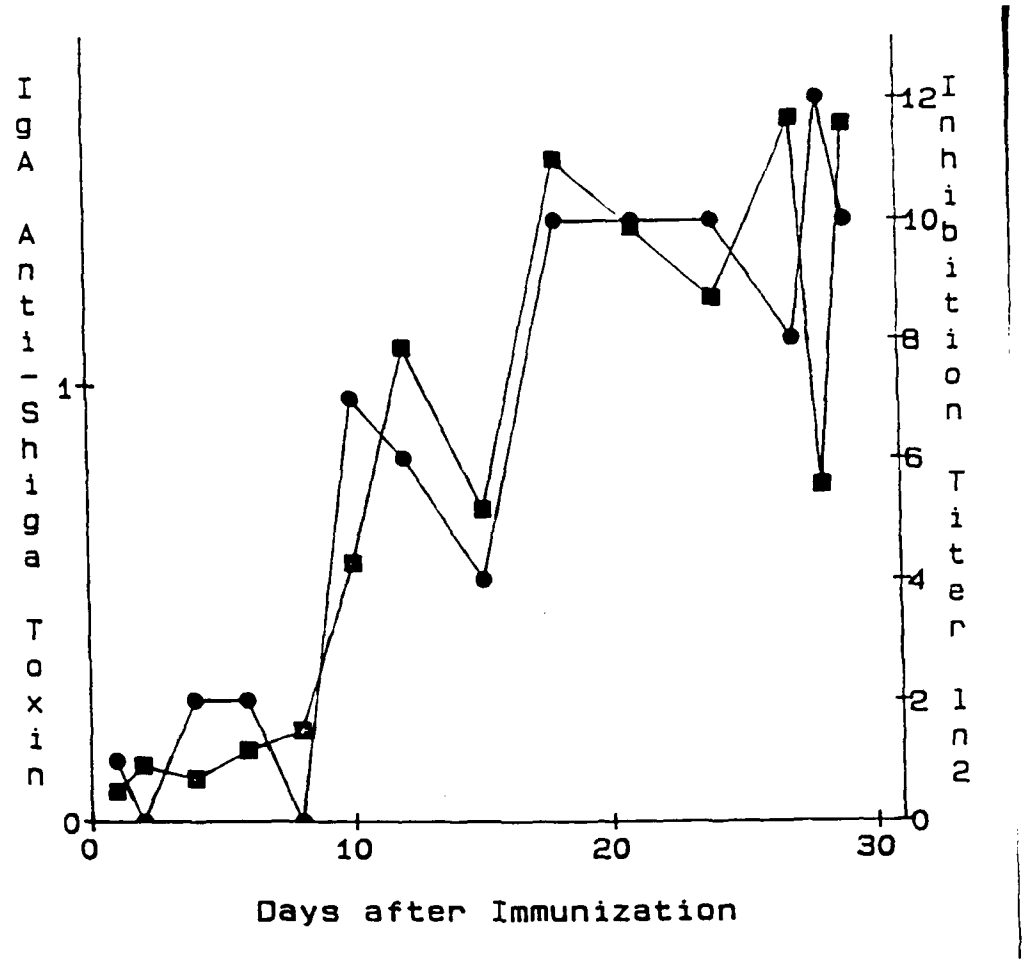

Fig. 4. Mean IgA anti-Shiga toxin activity (ELISA) (squares) correlates well with the mean cytotoxicity inhibition titer (circles) in secretions from immunized rabbits

SUMMARY

Mucosal immunity to some enteropathogens occurs naturally following infection. By learning how to optimize initiation of the mucosal immune response it will be possible to develop vaccines against a wide variety of enteropathogens and their toxic products. In the past few years, we have examined stimulation of the mucosal response to Shigella antigens. We have found that the mucosal memory response to Shigella LPS can be stimulated by oral immunization with live, but not with killed Shigella. This primes specific B lymphocytes which, following rechallenge, quickly migrate from the Peyer's patches to mesenteric lymph nodes, the spleen, and back to the Peyer's patches. We have found that the uptake of *S. flexneri* is the initial step in developing a mucosal immune response to Shigella. Whereas there is little difference between the initial uptake of virulent and avirulent bacteria by M cells, pathogenic strains of Shigella are able to replicate following their uptake by the specialized M cells located in the follicle-associated epithelium of the gut. This likely serves as the source of the ulcerative lesions found in dysentery. Lastly, we have detected a vigorous secretory IgA response to Shiga toxin. The titer of IgA activity to Shiga toxin from these loop secretions correlated well with the ability to prevent Shiga toxin cytotoxin effects *in vitro*. The extremely vigorous mucosal immune response to Shiga toxin makes this an attractive alternative to cholera toxin to potentiate the secretory IgA immune response.

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